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<b>(21) International Application Number:</b> PCT/US98/06690 <b>(22) International Filing Date:</b> 3 April 1998 (03.04.98) <b>(30) Priority Data:</b> 60/043,047 4 April 1997 (04.04.97) US <b>(71) Applicant:</b> THE IMMUNE RESPONSE CORPORATION [US/US]; 5935 Darwin Court, Carlsbad, CA 92008 (US). <b>(72) Inventor:</b> LUFTIG, Ronald, B.; 5120 Pike Drive, Metairie, LA 70003 (US). <b>(74) Agents:</b> STEINHARDT, Paul, C. et al.; Campbell & Flores LLP, Suite 700, 4370 La Jolla Village Drive, San Diego, CA 92122 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, B BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, G GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, K LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MV MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, T TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO pate (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian pate (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European pate (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, I LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, C CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending th</i> <i>claims and to be republished in the event of the receipt o</i> <i>amendments.</i>

**(54) Title:** NON-INFECTIOUS, PROTEASE DEFECTIVE HIV PARTICLES AND NUCLEIC ACID MOLECULES ENCODING THEREFOR**(57) Abstract**

This invention is directed toward mutated DNA, proteins, or protein fragments and particles from the L-2 cell line. The invention is also directed to diagnostic, prophylactic and therapeutic methods of making and using the DNA, protein and particles.

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## NON-INFECTIOUS, PROTEASE DEFECTIVE HIV PARTICLES AND NUCLEIC ACID MOLECULES ENCODING THEREFOR

This application is based on, and claims the benefit of, U.S. Provisional Application No. 60/043,047, filed April 4, 1997, and entitled NON-INFECTIOUS, PROTEASE DEFECTIVE HIV PARTICLES AND NUCLEIC ACID MOLECULES ENCODING THEREFOR.

BACKGROUND OF THE INVENTION

This invention relates to human immunodeficiency viruses in general and, more specifically to non-infectious vaccines for the treatment of human immunodeficiency disease.

AIDS is a lethal disease caused by human immunodeficiency viruses HIV-1 and HIV-2. This disease is characterized by a long, latent asymptomatic phase. During this phase the HIV particles are suppressed by the body's immune defense system. The next phase of the disease occurs when the body's immune system is no longer able to suppress the viral particles. At this stage of the disease, the HIV viral particles attack and destroy a key component of the body's immune defense system called CD4<sup>+</sup> cells. Once these cells are destroyed, the third and final phase of the disease occurs. During this last phase, the body is extremely susceptible to infections by many different kinds of diseases. These so-called secondary infections are the cause of death in many AIDS patients.

One of the central mysteries about AIDS is why HIV particles can exist in the body for so long, yet the patient remains asymptomatic. As a corollary to this riddle, there is no certain explanation why the immune system suddenly fails to be effective against the particles, thus enabling the second phase of the disease.

One theory for the sudden onslaught of the HIV particles on the CD4<sup>+</sup> cells is that this onslaught is made by "defective" HIV particles. These new, defective particles are not recognized by the body's immune system.

- 5 These defective particles have mutations in some of the key polypeptides of an HIV viral particle, yet somehow are still able to affect sufficient damage on the CD4<sup>+</sup> cells by means other than the classical invasion of the cells.

- 10 Surprisingly, non-infectious HIV particles produced by a cell called "L-2" fuse more efficiently than wild-type HIV with a subpopulation of CD4<sup>+</sup> cells. The enhanced fusion is caused by the 4-fold increased level of gp120, the two deletion mutations in gp120,  
15 altered conformational epitopes of gp120 and/or a combination of these factors, as well as mutant forms of Nef, gp41, protease, and Vpr, as discussed below. The fusion is far from the typical action of normal HIV infectious viral particles, in that it leads to the  
20 stimulation and release of IL-2R, Fas ligand and interferon-gamma 1 from a small subset of CD4<sup>+</sup> cells (Kameoka et al., International Immunology 9(10):1453-1462 (1997)). This occurs after L-2 particle fusion with these CD4<sup>+</sup> cells, a high percentage of uninfected, healthy  
25 neighboring CD4<sup>+</sup>, as well as CD8<sup>+</sup> cells, are prematurely thrown into a programmed cell death cycle, ultimately resulting in apoptosis. Apoptosis is a natural process in most cells in the body, but only after such cells have become senescent. Fusion of L-2 particles with CD4<sup>+</sup> cells  
30 can also cause such cells to form multinucleated protoplasmic masses called "syncytia", which die shortly thereafter.

- Thus, there exists a need to detect such defective HIV particles in the infected population; both  
35 for asymptomatic carriers, as well as for ARC (AIDS

Related Complex) or AIDS patients. Furthermore, a need exists for prophylactic, immunogenic, and therapeutic agents for such defective particles, as well as polypeptides or fragments of polypeptides derived from the particles or their mutant nucleic acid sequences. This need exists due to the fact that mutations found in L-2 particles are normally produced in HIV infected individuals, so that development of immunity to them by natural or artificial means is a key to thwarting the pathogenic process leading to ARC and AIDS. This invention provides these advantages and more.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 sets forth the mutations in the nucleotide sequences and deduced amino acid residues of the defective HIV-1 provirus in L-2 cells compared with those in MO/LAI and LAI cells. The portions of the nucleotide sequences of the relevant *pol* protease (prot.), *vpr*, *env* (gp120 and gp41), and *nef* gene regions in wild-type HIV-1 (LAI), as well as the proviruses of L-2 or MO/LAI, are shown in the upper part for each gene. The portions of the deduced amino acid sequences of Pol, Vpr, Env and Nef polypeptides setting forth the mutations are shown below the nucleotide sequences. The wild-type LAI control sequences are taken from previously reported sequences for HIV-1 (LAI), in GenBank. The sequences identical with those of wild-type LAI are indicated by dots. An asterisk (\*) indicates the appearance of stop codons. For the upper part, a dash (-) indicates a deletion in nucleotide sequences. For the amino acid sequences represented in the lower part, a dash indicates sequences identical with the wild type HIV-1 (LAI) sequence. A hatchmark (#) indicates a deletion in deduced amino acid residues. Three pluses (+) indicates any three nucleotides coding for the corresponding amino acid shown in the deduced amino acid sequence.

Figure 2 sets forth the primers used for PCR amplification of protease-defective HIV-1 provirus in L-2 cells. Nucleotide numbers are those of the HIV-1 molecular clone pNL432 (Adachi et al., J. Virol, 59:284-291 (1986)).

Figure 3 sets forth the partial nucleic acid sequence of the mutated *nef* gene of the HIV-1 protease-defective provirus in the L-2 cell, where the individual bases that differ from the wild-type LAI HIV-1 virus in GenBank are encircled.

Figure 4 sets forth the sequence of the truncated *Nef* polypeptide coded for by the mutated *nef* gene of the L-2 HIV-1 provirus. The encircled amino acids differ from the wild-type LAI HIV-1 virus amino acid sequence set forth in GenBank.

#### SUMMARY OF THE INVENTION

This invention is directed to nucleic acid molecules and fragments thereof containing mutations in certain genes of the HIV-1 virus, and the polypeptides and fragments thereof that are encoded by such mutated nucleic acid molecules, antibodies to such polypeptides, nucleic acid and antibody-based assays, as set forth below, as well as therapeutic uses of such antibodies. This invention is also directed to immunogens, and compositions of immunogens and adjuvants, containing such mutated HIV-1 viruses and in the production and use in therapeutic and prophylactic (immunogenic) methods and compositions.

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to the identification of mutations within pathologically important genes of the human immunodeficiency genome (HIV) which render viral particles non-infectious. The non-infectious phenotype of the viral particles can advantageously be used as a natural source of material for the production of immunogens for vaccination against or treatment of Acquired Immunodeficiency Syndrome (AIDS). The mutations identified within the various pathologically important genes can also be advantageously used to engineer new non-infectious viral particles for use as immunogens against essentially any type of HIV isolate.

In one embodiment, the invention is directed to the mutations discovered in various pathologically important genes of the non-infectious, protease-defective HIV-1 provirus harbored by L-2 cells. The partial sequences illustrating the nucleotide and amino acid changes caused by these mutations are set forth in Figures 1, 3 and 4.

The formation of syncytia and apoptosis of bystander T cells appear to be a mechanism of HIV-1-induced pathogenesis, leading to the diminution of T cells. (Amazon and Capon, Immunol. Today, 12:102-105 (1991); Malderelli et al., J. Virology, 69:6457-6465 (1995); and Sodroski et al., Nature, 322:470-474 (1996)). It has been reported that subclones obtained from surviving cells after MT-4, a CD4 human T cell line, had been infected with wild-type LAI or infectious molecular clone pNLA32-derived viruses, were predominantly producers of infectious but less cytopathogenic virus particles (Yunoki et al., Arch. Virol., 116:143-158 (1991); Nishino et al., Arch. Virol., 120:181-192 (1991)). The provirus in these persistently infected

cell clones carried mutations in accessory genes, such as *vif*, *vpr*, and *vpu*. (Nishino et al., J. Gen. Virol., 75:2241-2251 (1994), and Kishi et al., J. Virol., 69:7507-7518 (1995)). However, about 10% of the subclones produced above with LAI virus showed defective phenotypes even in essential gene products. In particular, one of these, named the L-2 cell clone, was a large-scale producer of protease-defective, gp120-containing noninfectious particles. (Ikuta et al., J. Cancer Res., 78:118-123 (1988)).

Surprisingly, the protease-defective viral particles produced from the L-2 cell clone rapidly induce syncytium formation of uninfected T cells by virus-to-cell fusion (fusion from without), without the need for viral replication. (Ohki et al., J. AIDS, 4:1233-1240 (1991)). In addition, the L-2 particles have a significantly higher (>fivefold) activity for apoptosis induction in primary peripheral blood T cells, as well as a certain subset of U937 cells when compared with soluble recombinant gp120 or the wild-type HIV-1 (LAI) strain (Kameoka et al., Int. Immunol., 11:1687-1697 (1996); Kameoka et al., J. Clin. Microbiol., 35:41-47 (1997)). Sodium dodecyl sulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis demonstrates that the Gag precursor polypeptide in L-2 particles was not cleaved to mature Gag polypeptides and that the Env/Gag ratio in L-2 particles was about fourfold higher than that in HIV-1 (LAI) wild-type particles produced from persistently infected MOLT-cells (Yunoki et al., Arch. Virol., 116:143-158 (1991); Kameoka et al. (1997) supra)).

"Substantially pure" when used to describe the state of the claimed nucleic acids, polypeptides or fragments thereof, is intended to mean that the claimed

molecules are free of at least a portion of the contents associated with or occurring with the claimed nucleic acids, polypeptides or fragments thereof in the native environment.

5           As used herein, the term "substantially" or "substantially the same" when used in reference to a nucleotide or amino acid sequence is intended to mean that the nucleotide or amino acid sequence shows a considerable degree, amount or extent of sequence  
10 identity-when compared to a reference sequence. Such considerable degree, amount or extent of sequence identity is further considered to be significant and meaningful and therefore exhibit characteristics which are definitively recognizable or known. Thus, a  
15 nucleotide sequence which is substantially the same nucleotide or amino acid sequence as a claimed sequence, refers to a sequence which exhibits characteristics that are definitively known or recognizable as representing the claimed nucleotide or amino acid sequence and minor  
20 modifications thereof.

In regard to substantially the same nucleotide sequences, such characteristics include for example, specific hybridization of a nucleotide sequence to a claimed nucleic acid or its complement. Hybridization  
25 principles and methods for determining hybridization specificity are well established and are known by one skilled in the art. Such principles and methods can be found in, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor  
30 Laboratory, New York (1992), and in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, MD (1989). Thus, it is not necessary that two nucleic acids exhibit sequence identity to be

substantially complimentary, only that they can specifically hybridize or be made to specifically hybridize without detectable cross reactivity with other similar sequences.

5           In regard to substantially the same amino acid sequence, such characteristics which are definitively recognizable or known include, for example, maintaining the biological function of the polypeptide or substitution of conservable amino acid residues. It is  
10 understood that limited modifications may be made without destroying the biological function of the claimed polypeptides or fragments thereof, and that only a portion of the entire primary structure can be required in order to effect activity. For example, the claimed  
15 polypeptides of the invention have an amino acid sequence substantially similar to those indicated for the L-2 polypeptides shown in Figures 1 or 4, but minor modifications of these sequences which do not destroy their activity also fall within the definition of the  
20 polypeptides claimed as such. Moreover, fragments of the sequences of the L-2 polypeptides shown in Figures 1 and 4, are similarly included within the definition. It is understood that minor modifications of primary amino acid sequence may result in polypeptides which have  
25 substantially equivalent or enhanced function as compared to the sequences of the L-2 polypeptides set forth in Figures 1 and 4. These modifications can be deliberate, as through site-directed mutagenesis, or may be accidental such as through mutation in hosts which  
30 produce the claimed polypeptides. All of these modifications are included as long as the biological functions are retained.

As used herein, the term "fragment" when used in reference to a nucleic acid encoding the claimed polypeptides is intended to mean a nucleic acid having substantially the same sequence as a portion of a nucleic acid encoding the claimed *Pol* protease, *Nef*, *Env* gp41, *Env* gp120 or *Vpr* polypeptides or fragments thereof. The nucleic acid fragment is sufficient in length and sequence to selectively hybridize to a *Pol* protease, *Nef*, *Env* gp41, gp120 or *Vpr* encoding nucleic acid or a nucleotide sequence that is complimentary to a *Pol* protease, *Nef*, *Env* gp41, gp120 or *Vpr* encoding nucleic acid. Therefore, fragment is intended to include primers for sequencing and polymerase chain reaction (PCR) as well as probes for nucleic acid blot or solution hybridization.

As used herein, the term "functional fragment" when used in reference to a *Pol* protease, *Nef*, *Env* gp41, gp120 or *Vpr* polypeptide is intended to refer to a portion of a *Pol* protease, *Nef*, *Env* gp41, gp120 or *Vpr* polypeptide which still retains some or all or the apoptotic inducing activity of the L2 particles containing one or more of these polypeptides as described herein. The term is also intended to include polypeptides that include, for example, modified forms of naturally occurring amino acids such as D-stereoisomers, non-naturally occurring amino acids, amino acid analogues and mimics so long as such polypeptides retain functional activity as defined above.

The HIV-1 provirus from L-2 cells has been analyzed with respect to genetic mutations that result in non-infectious HIV particles. Genes of HIV-1 provirus from L-2 cells have been isolated and sequenced and

compared to the wild type HIV-1 MO/LAI and LAI isolates. Various mutations have been observed in several viral genes encoding viral polypeptides, including the *nef*, *env*, *pol* and *vpr* genes.

5 One aspect of the instant invention is a substantially pure nucleic acid molecule encoding a mutant HIV-1 Nef protein, containing the encircled L-2 mutations set forth as SEQ ID NO: 45 (Fig. 3), and any fragments of such a nucleic acid molecule containing at  
10 least one of the encircled L-2 mutations which can be used to specifically detect the mutated nucleic acid or protein.

The *nef* gene of HIV-1 provirus from L-2 cells was compared to the wild type HIV-1 in MO/LAI and LAI  
15 isolates GenBank accession No. K02013. Several mutations in the *nef* gene compared to the wild type HIV-1 *nef* gene sequence were observed. One mutation is the substitution of A for G at nucleotide 169 of the *nef* gene. This  
20 mutation results in a stop codon at amino acid residue 57 such that the Nef polypeptide is truncated and contains amino acids 1 through 56.

The invention provides a substantially pure nucleic acid molecule or fragment thereof that encodes a truncated Nef protein comprising a nucleic acid sequence  
25 that encodes substantially the same sequence of amino acid residues 1 through 56 of an HIV-1 Nef polypeptide.

Another mutation found in the *nef* gene of HIV-1 provirus from L-2 cells is the substitution of G for the A at nucleotide 43 of the wild type *nef* gene. This  
30 mutation changes Thr at amino acid residue 15 of the Nef polypeptide to Ala. An additional mutation found in the

*nef* gene of HIV-1 provirus from L-2 cells is the substitution of A for the G at nucleotide 56 of the wild type *nef* gene. This mutation changes Arg at amino acid residue 19 of the Nef polypeptide to Lys.

- 5 Additional mutations of the *nef* gene of HIV-1 provirus from L-2 cells were found. One of these mutations is the substitution of T for the C at nucleotide 98 of the wild type *nef* gene. This mutation changes Ala at amino acid residue 33 of the Nef  
10 polypeptide to Val. Still another of these mutations is the substitution of A for the C at nucleotide 161 of the wild type *nef* gene. This mutation changes Ala at amino acid residue 54 of the Nef polypeptide to Asp.

- An additional mutation found in the *nef* gene of  
15 HIV-1 provirus from L-2 cells is the substitution of A for the G at nucleotide 111 of the wild type *nef* gene. This mutation results in no change in the amino acid at residue 37 of the encoded Nef polypeptide since the nucleotide substitution occurs in the third position of  
20 the codon for Leu and all nucleotide substitutions at this position would encode for Leu. Therefore, nucleotide 111 of the *nef* gene can be any of the four nucleotides.

- The invention also provides a substantially  
25 pure nucleic acid or fragment thereof that encodes a Nef polypeptide comprising a nucleotide sequence that encodes substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Ala at amino acid residue 15. The invention additionally provides a substantially pure  
30 nucleic acid or fragment thereof that encodes a Nef polypeptide comprising a nucleotide sequence that encodes

substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Lys at amino acid residue 19.

The invention further provides a substantially pure nucleic acid or fragment thereof that encodes a Nef polypeptide comprising a nucleotide sequence that encodes substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Val at amino acid residue 33. The invention also provides a substantially pure nucleic acid or fragment thereof that encodes a Nef polypeptide comprising a nucleotide sequence that encodes substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Asp at amino acid residue 54.

The nucleotide substitutions and amino acid mutations found in the *nef* gene of HIV-1 provirus from L-2 cells and the encoded Nef polypeptide are shown in Figures 3 and 4, respectively.

A second aspect of the instant invention is a substantially pure nucleic acid molecule encoding a mutated HIV-1 *env* gp41 protein containing the L-2 mutation set forth in SEQ ID NO: 29 (Fig. 1) and any fragment of such nucleic acid molecule containing the mutation which can be used to specifically detect the mutated nucleic acid or protein.

A mutation in the L-2 cell derived HIV-1 provirus *env* gene encoding the Env gp41 polypeptide has also been identified. The mutation is the substitution of G for the A at nucleotide 7781 (nucleotide 1979 in Figure 1) in a wild type *env* gene. This mutation changes Lys at amino acid residue 660 of the Env gp41 polypeptide to Arg.

The invention also provides a substantially pure nucleic acid or fragment thereof that encodes an Env gp41 polypeptide comprising a nucleotide sequence that encodes substantially the same amino acid sequence of an HIV-1 Env gp41 polypeptide containing Arg at amino acid residue 660.

A third aspect of the instant invention is a substantially pure nucleic acid molecule encoding a mutated HIV-1 *pol* protease protein containing the L-2 mutation set forth in SEQ ID NO:5 (Fig. 1) and any fragment of such nucleic acid molecule containing the mutation which can be used to specifically detect the mutated nucleic acid or protein.

A mutation in the L-2 cell derived HIV-1 provirus *pol* protease gene encoding the *pol* protease polypeptide has also been identified. The mutation is the insertion of T after the A at nucleotide 41 in wild type *pol* protease gene. This mutation results in a frameshift and the appearance of a stop codon at amino acid residue 30 of the Pol protease polypeptide. This results in a 29 amino acid Pol protease polypeptide with the carboxyl-terminal amino acid sequence AsnAspArgGlyAlaThrLysGlySerSerIleArgTyrArgSerArg (SEQ ID NO:6) starting at amino acid residue 14.

The invention additionally provides a substantially pure nucleic acid or fragment thereof that encodes a Pol protease polypeptide comprising a nucleotide sequence that encodes substantially the same sequence of amino acid residues 1 through 13 of an HIV-1 Pol protease polypeptide fused at the carboxyl-terminus to substantially the same amino acid sequence

AsnAspArgGlyAlaThrLysGlySerSerIleArgTyrArgSerArg (SEQ ID NO:6).

A fourth aspect of the invention is directed to a substantially pure nucleic acid molecule encoding a mutant HIV-1 gp120 protein containing the L-2 mutations set forth in SEQ ID NOS:17 and 23 (Fig. 1), and any fragment of said nucleic acid containing one or more of the L-2 mutations which can be used to specifically detect the mutated nucleic acid or protein.

10 Mutations in the L-2 cell derived HIV-1 provirus env gene encoding the Env gp120 polypeptide have also been identified. A mutant Env gp120 found in HIV-1 provirus from L-2 cells has a substitution of Arg for the Ser at amino acid residue 143, substitution of Ile for  
15 the Met at position 153 and deletion of eight amino acids, from 144 through 151, of the wild type HIV-1 from LAI cells as well as substitution of Val for the Ile at amino acid residue 187 and deletion of two amino acids, 192 and 193, of the wild type HIV-1 from LAI cells. The  
20 substitution of Arg at amino acid position 143 occurred due to nucleotide substitutions that changed the codon for Ser to the codon for Arg. Any mutation that results in changing the nucleotides encoding Ser 143 (nucleotides 427 through 429) to Arg can be used in the nucleic acids  
25 of the invention. For example, nucleotides 427 through 429, AGT, can be substituted with nucleotides that result in a codon for Arg, such as substitution of A for the T at nucleotide 429, substitution of G for the T at nucleotide 429, substitution of C for the A at nucleotide  
30 427, substitution of C for the A at position 427 combined with substitution of C for the T at nucleotide 429, substitution of C for the A at position 427 combined with the substitution of A for the T at nucleotide 429, and

substitution of C for the A at position 427 combined with the substitution of G for the T at nucleotide 429, all of which result in substitution of Arg for Ser at amino acid position 143. The substitution of Ile for Met at amino acid residue 153 results from substitution of A for the G at nucleotide 459. The substitution of Val for Ile at amino acid residue 187 results from substitution of G for A at nucleotide 559. This mutant Env gp120 mutant is identical to a Env gp120 mutant found in HIV-1 from MO/LAI (see Figure 1).

The invention also provides a substantially pure nucleic acid or fragment thereof that encodes an Env gp120 polypeptide comprising a nucleotide sequence that encodes substantially the same amino acid sequence of an HIV-1 Env gp120 polypeptide containing Arg at amino acid residue 143, Ile at amino acid residue 153, and Val at the amino acid residue corresponding to amino acid residue 187 of a wild type HIV-1 Env gp120 polypeptide and absent amino acid residues corresponding to amino acid residues 144 through 151 and 192 through 193 of a wild type HIV-1 Env gp120 polypeptide.

A fifth aspect of the invention is directed to a substantially pure nucleic acid molecule encoding a mutant HIV-1 Vpr protein containing the L-2 mutation set forth in SEQ ID NO: 11 (Fig. 1), and any fragment of such a nucleic acid molecule containing the L-2 mutation which can be used to specifically detect the mutated nucleic acid or protein.

A mutation in the L-2 cell derived HIV-1 provirus vpr gene encoding the Vpr polypeptide has also been identified. The mutation is the substitution of A for the G at nucleotide 5194 (nucleotide 54 in Figure 1)

in a wild type vpr gene. This mutation introduces a stop codon at amino acid residue 18 such that the vpr gene encodes a truncated Vpr polypeptide containing amino acid residues 1 through 17.

5           The invention also provides a substantially pure nucleic acid or fragment thereof that encodes a Vpr polypeptide comprising a nucleotide sequence that encodes substantially the same sequence of amino acid residues 1 through 17 of an HIV-1 Vpr polypeptide.

10           In addition to the nucleic acids described above encoding individual Nef, Env gp41, Pol protease, Env gp120 and Vpr polypeptide mutants, the invention also provides substantially purified nucleic acids encoding combinations of these mutant polypeptides. In one  
15   embodiment, any of the mutations in an individual polypeptide can be used alone or in combination with one or more additional mutations in the same polypeptide. Thus, the invention provides a substantially pure nucleic acid or fragment thereof that encodes a HIV-1 polypeptide  
20   having one or more of the mutations described above.

          For example, a nucleic acid encoding a Nef polypeptide can include the deletion mutant, which encodes amino acid residues 1 through 56 of the Nef polypeptide, and any combination of one or more of the  
25   amino acid substitutions. The invention provides a nucleic acid encoding the truncated Nef polypeptide containing amino acid residues 1 through 56 and Ala at amino acid residue 15. A nucleic acid can also encode the truncated Nef polypeptide with Lys at amino acid  
30   residue 19. Similarly, any of the amino acid substitution mutations in the Nef polypeptide described above can be combined with the truncated Nef polypeptide.

Alternatively, any of the amino acid substitution mutations can be combined with each other in the full length Nef polypeptide or a fragment thereof.

The invention thus provides a substantially  
5 pure nucleic acid or fragment thereof that encodes at least one of the mutations described above and can, where more than one mutation has been described in an individual polypeptide, contain any combination of two or more mutations, including as many as all of the mutations  
10 described for an individual polypeptide. An example of such a nucleic acid encoding an individual polypeptide having multiple mutations is shown in Figure 3 (SEQ ID NO:45).

In another example of a nucleic acid encoding  
15 an individual polypeptide for which multiple mutations have been identified, a nucleic acid encoding Env gp120 can contain the deletion of up to eight amino acids of amino acid residues 144 through 151. A nucleic acid encoding Env gp120 can also contain the deletion of one  
20 or two amino acids of amino acid residues 192 and 193. A nucleic acid encoding Env gp120 can additionally contain the combination of deletion of up to eight amino acids of amino acid residues 144 through 151 and deletion of one or two amino acids of amino acid residues 192 and 193.  
25 Additionally, any combination of one or both of the deletion mutations can be combined with Arg at amino acid residue 143. The combination of one or both of the deletion mutations can also be combined with Ile at amino acid residue 153. Also, the combination of one or both  
30 of the deletion mutations can be combined with Val at amino acid residue 187. Additionally, the combination of one or both of the deletion mutations can be combined with one or two of the single amino acid substitutions.

In another embodiment in which nucleic acids encode a combination of amino acid mutations of polypeptides, the invention also provides a substantially pure nucleic acid or fragment thereof that encodes more than one polypeptide containing at least one mutation. A nucleic acid of the invention can therefore encode any one or more of the polypeptides selected from the group consisting of Nef, Env gp41, Pol protease, Env gp120 and Vpr polypeptides so long as the encoded polypeptides contain at least one of the mutations described above. For example, a substantially pure nucleic acid can encode a truncated Nef polypeptide containing amino acid residues 1 through 56 and Env gp41 containing Arg at amino acid residue 660. In another embodiment, a substantially pure nucleic acid can encode three or more of the polypeptides selected from the group consisting of Nef, Env gp41, Pol protease, Env gp120 and Vpr polypeptides, up to and including all of these polypeptides so long as at least one of the amino acid mutations described above is encoded by the nucleic acid. For example, a substantially pure nucleic acid can encode all of the polypeptides Nef, Env gp41, Pol protease, Env gp 120 and Vpr polypeptides having all of the amino acid mutations described above.

Further aspects of this invention are the substantially pure polypeptides or fragments thereof that are encoded by any of the above nucleic acid molecules. In one aspect of the invention, the substantially pure polypeptides or fragments thereof can be used to raise antibodies which are specific to the mutated proteins.

The invention provides a substantially pure HIV-1 polypeptide or a fragment thereof selected from the group of polypeptides consisting of Nef, Env gp41, Pol

protease, Env gp120 and Vpr, wherein the HIV-1 polypeptide contains at least one of the mutations found in HIV-1 provirus from L-2 cells. Such mutations found in HIV-1 provirus from L-2 cells are described above.

5           In one embodiment, the invention provides Nef polypeptides having one or more mutations in the Nef polypeptide of a HIV-1 provirus from L-2 cells. For example, the invention provides a substantially pure Nef polypeptide or fragment thereof comprising substantially  
10 the same sequence of amino acid residues 1 through 56 of a HIV-1 Nef polypeptide. The invention also provides a substantially pure Nef polypeptide or fragment thereof comprising substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Ala at amino acid  
15 residue 15. The invention additionally provides a substantially pure Nef polypeptide or fragment thereof comprising substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Lys at amino acid residue 19.

20           The invention further provides a substantially pure Nef polypeptide or fragment thereof comprising substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Val at amino acid residue 33. The invention also provides a substantially pure Nef  
25 polypeptide or fragment thereof comprising substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Asp at amino acid residue 54.

          In addition to the Nef polypeptides having the individual mutations described above, the invention also  
30 provides a substantially pure Nef polypeptide or fragment thereof having any combination of one or more of the Nef polypeptide mutations. For example, the invention

provides Nef polypeptides having one or more of the amino acid substitution mutations described above in the full length Nef polypeptide. Furthermore the invention provides a truncated Nef polypeptide and a combination of one or more of the amino acid substitution mutations described above. For example, the invention provides a truncated Nef polypeptide containing amino acids 1 through 56 and Ala at amino acid residue 15. Additionally, the invention provides a truncated Nef polypeptide containing amino acids 1 through 56 and Lys at amino acid residue 19. Further provided is a truncated Nef polypeptide containing amino acids 1 through 56 and Val at amino acid residue 33. Also provided is a truncated Nef polypeptide containing amino acids 1 through 56 and Asp at amino acid residue 54.

In addition to the Nef polypeptide truncation mutants combined with a single amino acid substitution mutation, the invention also provides a truncated Nef polypeptide containing amino acids 1 through 56 and two or more of the amino acid substitution mutations described above. For example, the Nef polypeptide can contain amino acid residues 1 through 56 and Asp at amino acid residue as well as Val at amino acid residue 33. Furthermore, the invention provides a truncated Nef polypeptide containing amino acid residues 1 through 56 as well as Ala at amino acid residue 15, Lys at amino acid residue 19, Val at amino acid residue 33, and Asp at amino acid residue 54. Therefore, one aspect of the instant invention is the truncated HIV-1 Nef protein containing the amino acid substitutions set forth in SEQ ID NO: 46 (Fig. 4). The amino acid sequence of such a Nef polypeptide combining all of the Nef polypeptide mutations found in HIV-1 provirus from L-2 cells is shown in Figure 4 (SEQ ID NO:46).

The invention also provides a substantially pure Env gp41 polypeptide or fragment thereof comprising substantially the same amino acid sequence of an HIV-1 Env gp41 polypeptide containing Arg at amino acid residue 5 660. The invention additionally provides a substantially pure Pol protease polypeptide or fragment thereof comprising substantially the same sequence of amino acid residues 1 through 13 of an HIV-1 Pol protease polypeptide fused at the carboxyl-terminus to 10 substantially the same amino acid sequence as AsnAspArgGlyAlaThrLysGlySerSerIleArgTyrArgSerArg (SEQ ID NO:6).

The invention also provides a substantially pure Env gp120 polypeptide or fragment thereof comprising 15 substantially the same amino acid sequence of an HIV-1 Env gp120 polypeptide containing Arg at amino acid residue 143, Ile at amino acid residue 153, and Val at the amino acid residue corresponding to amino acid residue 187 of a wild type HIV-1 Env gp120 polypeptide 20 and absent amino acid residues corresponding to amino acid residues 144 through 151 and 192 through 193 of a wild type HIV-1 Env gp120 polypeptide.

The invention also provides a substantially pure Env gp120 polypeptide comprising substantially the 25 same amino acid sequence of an Env gp120 polypeptide with deletion of at least one and up to eight amino acids of amino acid residues 144 through 151. The invention additionally provides a substantially pure Env gp120 polypeptide comprising substantially the same amino acid 30 sequence of an Env gp120 polypeptide with deletion of one or both of amino acid residues 192 and 193.

Additional Env gp120 polypeptides are also provided in the present invention. For example, the invention provides an Env gp120 polypeptide comprising deletion of at least one and up to eight amino acids of amino acid residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193. Additionally, the invention provides Env gp120 polypeptides comprising deletion of at least one and up to eight amino acids of amino acid residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193 as well as Arg at amino acid residue 143. The invention also provides Env gp120 polypeptides comprising deletion of at least one and up to eight amino acids of amino acid residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193 as well as Ile at amino acid residue 153. Also, the invention provides Env gp120 polypeptides comprising deletion of at least one and up to eight amino acids of amino acid residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193 as well as Val at amino acid residue 187.

The invention further provides Env gp120 polypeptides comprising deletion of at least one and up to eight amino acids of amino acid residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193 as well as two of the amino acid substitution mutations. For example, the invention provides Env gp120 polypeptides comprising deletion of at least one and up to eight amino acids of amino acid residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193 as well as Arg at amino acid residue 143 and Ile at amino acid residue 153. The invention also provides Env gp120 polypeptides comprising deletion of at least one and up to eight amino acids of amino acid

residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193 as well as Arg at amino acid residue 143 and Val at amino acid residue 187.

The invention further provides Env gp120 polypeptides  
5 comprising deletion of at least one and up to eight amino acids of amino acid residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193 as well as Ile at amino acid residue 153 and Val at amino acid residue 187.

10 The invention also provides a substantially pure Vpr polypeptide or fragment thereof comprising substantially the same sequence of amino acid residues 1 through 17 of an HIV-1 Vpr polypeptide.

As discussed above for Nef polypeptide and Env  
15 gp120, any of the mutations in an individual polypeptide can be used alone or in combination with one or more additional mutations in the same polypeptide. Thus, the invention provides a substantially pure HIV-1 polypeptide or fragment thereof selected from the group consisting of  
20 Nef, Env gp41, Pol protease, Env gp120 and Vpr that contain two or more of the mutations described above in a single individual polypeptide.

The invention thus provides a substantially pure HIV-1 polypeptide or fragment thereof selected from  
25 the group of polypeptides consisting of Nef, Env gp41, Pol protease, Env gp120 and Vpr, wherein the HIV-1 polypeptide or fragment thereof comprises at least one of the mutations described above and can, where more than one mutation has been described in an individual  
30 polypeptide, comprise any combination of two or more mutations, including as many as all of the mutations described for an individual polypeptide.

Any combination of one or more of the HIV-1 polypeptides containing any combination of one or more of the mutations described above can be expressed together as desired. The choice of combination of polypeptides  
5 and the combination of mutations in the polypeptides depends on the needs of the investigator.

The invention also provides nucleic acids encoding substantially the same amino acid sequence of the mutant HIV-1 polypeptides described above. Any  
10 nucleic acid that encodes any combination of single mutations in a polypeptide, multiple mutations in a polypeptide or a combination of two or more polypeptides of the invention are also provided.

The genes of HIV-1 provirus from L-2 cells  
15 described above were isolated using PCR amplification as described in Example I. However, the nucleic acid molecules described above can be isolated from L-2 cells by any of a variety of methods well known in the art. For example, in addition to PCR amplification of nucleic  
20 acids, other methods well known in the art regarding cloning of DNA from an organism can be used to isolate HIV-1 provirus DNA (Sambrook et al., Eds., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Springs Harbor Laboratory Press (1989), which is herein  
25 incorporated by reference). Furthermore, the nucleic acids of the invention, which are set forth above, can be synthesized by chemical means (See, Blackburn and Gait, Eds., Nucleic Acids in Chemistry and Biology, 2nd Ed., Oxford University Press (1996), which is herein  
30 incorporated by reference).

The proteins and peptides encoded by said nucleic acids can be isolated and purified from L-2 viral

particles (see, for example, Deutscher, Ed., Methods in Enzymology: Guide to Protein Purification, Vol. 182, Academic Press, Inc., San Diego, CA (1990), herein incorporated by reference), or chemically synthesized or  
5 expressed in a recombinant organism by methods well known in the art, such as Stewart and Young, Eds., Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Company (1984); Kriegler, M., Ed., Gene Transfer and Expression: A Laboratory Manual, Stockton Press, New York, NY (1990);  
10 and Jones, J., Ed., The Chemical Synthesis of Peptides, Oxford University Press, Oxford (1994), all of which are herein incorporated by reference. For example, the substantially pure nucleic acids of the invention can be expressed recombinantly in bacterial or eukaryotic cells  
15 and subsequently purified.

As non-infectious particles, the L-2 viral particles can be used as immunogens to induce a host immune response against an HIV infection. More specifically, the immunogens can be used for the  
20 prevention or reduction of apoptotic cell lysis caused by HIV infection.

The L-2 viral particles can be produced by methods well known in the art. For example, non-infectious viral particles can be produced by growing L-2  
25 cells in culture under conditions such that protease-defective L-2 HIV particles are produced. Such particles are then separated from L-2 cells by, for example, centrifugation of the L-2 cell supernatant and recovering the particles in the pellet. Such methods are described  
30 further below in the Examples as well as in, for example, Ohki et al., J. Acquired Immun. Def. Syn. 4:1233-1240 (1991).

The non-infectious L-2 viral particles can be used directly as an immunogen or, alternatively, can be further treated to ensure multiple, additional levels of viral particle inactivation. Additional inactivation treatment is a precautionary measure to provide further confidence and safety levels against possible commination of non-L-2 HIV viral particles. Methods of viral particle inactivation are well known in the art and include, for example, treatment with gamma-radiation, beta-propiolactone and gluaraldehyde. These methods are described further below in the Examples. Moreover, the production and use of immunogens is taught, for example, in Salk et al., U.S. Patent No. 5,256,767, issued October 26, 1993. Therefore, the invention provides an immunogen consisting of non-infectious, protease-defective particles produced by L-2 cells. The invention also provides inactivated, protease-defective particles produced by L-2 cells. The non-infectious, protease-defective particles can also be produced by non-L-2 cells which harbor the L-2 provirus genome.

As described above, the L-2 viral particles contain a number of mutations which render the particles non-infectious. These mutations can be advantageously exploited to engineer a variety of non-infectious viral particles as specific immunogens against preselected types of HIV viruses. For example, it is not necessary to have all of the mutations described herein for the particle to be non-infectious and therefore useful as an immunogen. Instead, immunologically effective immunogens can be prepared from modified forms of the L-2 viral particles containing less than all of the mutations described herein so long as the modified L-2 viral particle contains a mutation that renders the particle non-infectious. Such mutations can be, for example, any

one of the mutations identified in the *Pol* protease, *Nef*, or *Env* gp41, for example. These mutations can be combined with the L-2 *Env* gp120 polypeptide or the L-2 *Env* gp120 mutants described herein to produce non-infectious viral particles for vaccinating against HIV. Other mutant polypeptides such as the L-2 *Vpr* or the L-2 *Vpr* mutants described herein can also be included within the viral particles to augment the non-infectious phenotype of the modified L-2 viral particles. Those skilled in the art will know or can determine which mutation or combination of mutations are sufficient to render the particle non-infectious given the mutant sequences and teachings described herein.

Alternatively, the L-2 mutations identified in the *Pol* protease, *Nef*, or *Env* gp41 polypeptides can be combined with *Env* gp120 polypeptides from other HIV virus sub-types, or clades, to produce non-infectious viral particles. These particles can similarly be used as vaccines against the various HIV clades. For example, there are two primary groups of HIV clades, termed M and O, which characterize the infective phenotype of the virus. For group M, there are eight clades (A through J). The L-2 viral particles are characterized as being within the B clade of Group M, Betts et al., J. Virology 71:8909-8911 (1997).

The L-2 viral particle are characterized as being within the O clade having subgroup E. Substituting *Env* gp120 derived from different clades or clade subgroups into non-infectious L-2 viral particles will render a modified L-2 particle specific for the substituted *Env* gp120 clade. These clade-specific, modified L-2 particles can then be used as immunogens for the prevention and treatment of an HIV infection.

As with the modified L-2 viral particles described above, immunogenically effective immunogens can be prepared from the clade-specific forms of the modified L-2 viral particles that contain less than all of the L-2 mutations described herein so long as the particles contain at least one of the mutations that renders the particle non-infectious. Such mutations can be, for example, any one of the mutations identified in the *Pol* protease, *Nef*, or *Env* gp41, for example. Additionally, the mutations described in the *Vpr* protein can be optionally incorporated. Those skilled in the art will know or can determine which mutation or combination of mutations are sufficient to render the clade-specific viral particles non-infections given the mutant sequences and teachings described herein.

Production of the modified L-2 particles or the modified, clade-specific particles described above can be performed using methods well known in the art given the L-2 nucleotide and amino acid sequences and the teachings described herein. Such methods can include, for example, the construction and expression of recombinant modified L-2 genomes so as to contain a desired combination of the L-2 mutations which confer the non-infectious phenotype. The construction of such desired combinations can be performed by, for example, site directed mutagenesis or by polymerase chain reaction (PCR) using mutagenic primers. Other well known methods exist in the art and can similarly be used to generate the desired combination of L-2 mutations within to produce a modified L-2 genome or a modified, clade specific genome. Once produced, the genomes can be stably introduced into compatible cells, such as T cells, to harbor the provirus and effect the production of the modified viral particles. Such methods are well known in the art and can be found described in,

for example, Sambrook et al., Eds., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press (1989), and Ausubel et al., Eds., Current Protocols in Molecular Biology, John Wiley & Sons, (1987). The

5 non-infectious, modified L-2 particles, or the non-infectious, modified particles that exhibit clade-specific phenotypes can be isolated and used directly as immunogens or be subjected to further inactivation procedures as described above prior to use as immunogens

10 for the prevention or treatment of apoptotic cell lysis mediated by HIV.

Specific examples of non-infectious, modified L-2 viral particles include particles containing at least an expressible *Nef* or *Pol* protease mutant as described

15 previously. For example, the *Nef* mutant can contain the stop codon at position 57 and/or contain one or more of the nucleotide and corresponding amino acid mutations set forth in Figures 3 and 4, (SEQ ID NOS:45 and 46, respectively). The *Pol* protease mutant can contain one

20 or more of the nucleotide and corresponding amino acid mutations set forth in Figure 1 (SEQ ID NO:5 and 6, respectively). Any of the *Nef* or *Pol* protease mutants can additionally be combined with the various *Vpr*, *Env* gp41 or *Env* gp120 mutants previously describe to generate

25 a non-infectious, modified L-2 viral particle of the invention. Similarly, such mutations can be constructed with non-L-2 *Env* gp120 polypeptides to produce non-infectious, modified L-2 particles that are clade specific.

30 Any of the previously described non-infectious L-2 viral particle immunogens, including modified forms and clade-specific forms, can additionally be combined with other HIV immunogens to further augment an immune

response directed to HIV. Additional HIV immunogens can include, for example, inactivated forms of the virus, virus which is devoid of its outer envelope proteins, as well as soluble HIV polypeptides or fragments thereof.

5 These additional HIV immunogens that can be combined with the non-infectious L-2 particles and modified forms thereof, can be derived from HIV-1, HIV-2 or both, for example. Such combined immunogens can similarly be formulated in an adjuvant for administration. Immunogens

10 for the induction of an immune response against HIV are well known in the art. A specific example of an immunogen which is devoid of the *Env* gp120 polypeptide is described, for example, in Salk et al., supra.

Therefore, the invention provides a method for

15 the reduction or prevention of apoptotic cell lysis mediated by HIV. The method consists of administration of a non-infectious L-2 particle or non-infectious modified L-2 particle to a human who is either seropositive or seronegative for HIV, and particularly in

20 those patients where the apoptotic lysis is induced by protease-defective particles. Additionally, the invention provides for methods of stimulating the immune system of a human. Such a method would comprise administering one of the above non-infectious L-2

25 particle or modified L-2 particle immunogens, or a composition including such immunogens in an immunologically effective amount to a human. Again, the human could be seronegative or seropositive for HIV.

The non-infectious immunogens of the invention

30 are administered to a host exhibiting or at risk of exhibiting an autoimmune response. Definite clinical diagnosis of AIDS or AIDS related complex (ARC) warrants the administration of the relevant immunogen.

Prophylactic applications are warranted where an HIV infection precedes the onset of overt clinical disease or where the individual has been at risk of exposure to the virus. Thus, individuals with any history of infection  
5 or predicted to be at risk by reliable prognostic indicators can be treated prophylactically to interdict immunodeficiency mechanisms prior to their onset.

The non-infectious immunogens of the invention can be administered in many possible formulations,  
10 including pharmaceutically acceptable mediums. The immunogens can include or be administered in conjunction with an adjuvant, of which several are known to those skilled in the art. After initial immunization with the non-infectious immunogen, further boosters can  
15 additionally be provided. The non-infectious immunogens are administered by conventional methods, in dosages which are sufficient to elicit an immunological response. Such dosages are known and also can be easily determined by those skilled in the art.

20 Briefly, individuals who are candidates for immunization can be effectively treated by active immunotherapy using a non-infectious immunogen prepared from the L2-viral particles or modified forms thereof. The dose is selected so as to be immunologically  
25 effective, and is generally between about 1 to about 100  $\mu\text{g}$  of protein, more preferably about 30  $\mu\text{g}$  of protein.

Active immunization is implemented and preferably repeated once at a minimum interval of at least 90 days, although additional boosts may be  
30 appropriate according to changes in the immunocompetence level, based, for example, on a decline in antibodies to HIV gene products other than outer envelope proteins.

Such immunization is preferably accomplished initially by intramuscular injection followed by intradermal injection, although any combination of intradermal and intramuscular injections may be used.

5 Preferably, the immunoresponsiveness or immunocompetence of the seropositive individual is determined prior to immunization in order to determine an appropriate course of therapy. As a method of such determination, individuals' sera can be screened for the  
10 presence of antibodies to p24 (as by means of ELISA), for TRI antibody and/or for the level of T<sub>H</sub> cells by methods well known in the art. Individuals exhibiting indicators of low immunocompetence, such as low p24 or RTI antibody titers or low numbers of T<sub>H</sub> cells, are appropriate  
15 candidates for active immunotherapy. Additionally, active immunotherapy can be combined with other therapies known in the art including, for example, passive immunotherapy.

Seronegative individuals can be vaccinated in  
20 order to induce immunoprotective factors to prevent infection. Preferably, the vaccine is administered initially by intramuscular injection followed by a booster injection given either intramuscularly or intradermally. A physiologically effective dose,  
25 preferably in the range of 1 to 100 µg and more preferably about 30 µg of immunogen is provided per dose. The vaccine can be administered in conjunction with an adjuvant, such as a water-in-oil type adjuvant. Various appropriate adjuvants are well known in the art as  
30 reviewed by Warren and Chedid, CRC Critical Reviews in Immunology 8:83 (1988).

These methods as well as other modes of administration are well known in the art and can be used for the prevention or treatment of an HIV infection using the non-infectious immunogens described herein.

5           The nucleic acid molecules discussed above can be used as nucleic acid probes or standards in either diagnostic or in PCR procedures. Such diagnostic methods can be used to detect the previously described mutant DNA sequences in cells and extracellular fluids. Methods of  
10 detection by hybridization with nucleic acid probes are well known in the art and are described in, for example, Hames and Higgins, Eds., Nucleic Acid Hybridisation: A Practical Approach, Oxford University Press, Oxford (1991), and Innis et al., Eds., PCR Protocols: A Guide  
15 to Methods and Applications, Academic Press, Inc. (1990). Various formats known in the art can be used for detection of L-2 specific mutations by nucleic acid hybridization. The choice of such formats will vary depending on the particular application and need of the  
20 diagnostic procedure. Formats include, for example, solid-phase hybridization and solution hybridization. Specific examples of solid-phase hybridization formats for diagnostic procedures include the use of diagnostic chips containing two-dimensional arrays of  
25 oligonucleotides, multi-well formats such as an ELISA and blot hybridization procedures. Such formats and various permutations thereof are well known in the art.

Similarly, the polypeptides above could be used as assay standards in immunoassays kits, and could be  
30 employed to raise antibodies for immunoassays to detect the above mutated polypeptides and organisms containing them. Methods for raising monoclonal and polyclonal antibodies specific to such polypeptides are well known

in the art. (See, for example, Harlow and Lane, Eds., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory (1988). Moreover, the production of monoclonal antibodies is described in detail in U.S. Patent Nos. RE 32011 and 4,411,993. Antibodies to L-2 mutant polypeptides can additionally be generated using combinatorial methods well known in the art. Such methods include the generation and screening of recombinant antibody library repertoires for antibodies specific to an antigen of interest, including any of the L-2 mutant polypeptides described herein (see, for example, Huse et al., Science 246:1275-1281 (1989)). Monoclonal antibodies specific for the L-2 polypeptide described herein can additionally be modified by recombinant methods known in the art to incorporate human antibody framework sequences. Such methods are well known in the art and are described in, for example, U.S. Patent No. 5,585,089 to Queen et al.

Once suitable antibodies or binding proteins have been obtained, they may be isolated or purified by many techniques well known to those of ordinary skill in the art (see Antibodies: A Laboratory Manual, Harlow and Lane (eds.), supra). Suitable techniques include peptide or protein affinity columns, HPLC or RP-HPLC, purification on protein A or protein G columns, or any combination of these techniques.

Therefore, the invention provides monoclonal and polyclonal antibodies, and fragments thereof specific for L-2 mutant polypeptides, as well as chimeric and humanized antibodies and fragments specific for such polypeptides. Such antibodies can be additionally modified with, for example, a label or a therapeutic agent for the diagnosis or treatment of HIV.

The antibodies raised against the above-described L-2 proteins can be used in quantitative, semi-qualitative, or qualitative diagnostic assays. Such assays, for example, can be in RIA, IRMA, and ELISA formats. Examples of such formats include a competitive or sandwich arrangement. See, for example, David et al., U.S. Patent No. 4,486,530, issued December 4, 1984; Motagnier et al., U.S. Patent No. 4,708,818, issued November 24, 1987; Montagnier et al., U.S. Patent No. 5,135,864, issued August 4, 1993; Gallo et al., U.S. Patent No. 4,647,773, issued March 3, 1987; Gallo et al., U.S. Patent No. 4,520,113, issued May 28, 1985; and Gallo et al., U.S. Patent No. 4,652,599, issued March 24, 1987, all of which are herein incorporated by reference. In a competitive format, the instant antibodies are incubated with the sample to be analyzed and a measured quantity of labeled, competing analyte. The antibody in competitive assay is usually bound to a solid support, or is capable of being bound to such a support, as discussed below.

Such binding to a solid support can take place before or after the instant antibody is incubated with the sample suspected of containing one of the instant proteins. In a sandwich format, two antibodies, each specific for the analyte, are reacted one at a time or simultaneously with the sample to be analyzed. Typically, one of the instant antibodies will be either be labeled with a detection means or capable of being so and the other will either be attached to a solid support or capable of being so. After the antibody-analyte-antibody sandwich complex is formed, the detection means is measured. Alternatively, after the complex is formed, the complex is then attached to a solid support or to a detection means, or both.

Binding to the solid support can be accomplished by an antibody that in turn binds to one of

the antibodies of the complex, the natural or covalent attachment of the unlabeled antibody to the support, or by the reaction of another ligand-receptor pair, such as biotin-avidin. In the latter case, biotin is bound  
5 either to the constant region of the antibody or to the solid support and avidin is bound to the remaining component. In either the competitive or sandwich format, there are usually wash, aspiration and possibly filter procedures after the incubation of the sample and  
10 following the additions of each reagent.

The antibodies used in the above diagnostic assays can use, for example, one or more of the instant polyclonal or monoclonal antibodies, mixtures thereof, and the corresponding functional fragments. For example,  
15 in a sandwich assay, the two antibodies used to form the complex can either both be polyclonal or monoclonal or functional fragments thereof. Alternatively, one monoclonal and one polyclonal can form the sandwich. Similarly, in the competitive format, the antibody can be  
20 one or more monoclonal or polyclonal antibodies, or functional fragments thereof.

The detection means are well known in the art. Such detection means can be, for example, enzymes, radioisotopes, fluorogens, chromogens, metallic or  
25 non-metallic colloids, colored liposomes, colored or colorable particles, and the like. Thus, the detection means may be immediately visible after the analyte complexes with the instant antibodies in an assay, or further steps may need to be taken to utilize the  
30 detection means. Such steps include UV and visible spectrophotometry, fluorimetry and analysis by radiation counters. Examples of such detection means include alkaline phosphatase/para-nitrophenyl phosphate; horse

radish peroxidase/aminoethylcarbazole, colloidal gold, colloidal silver, colloidal selenium, hydrophobic dyes, colored latex microparticles, carbon sols, rhodamine, fluorescein, luminol, luciferin, umbeliferone, <sup>125</sup>Iodine, <sup>131</sup>Iodine, tritium, <sup>32</sup>Phosphorous, and the like. These  
5 detection means can be coupled to either the analyte (competitive assays) or to an antibody (sandwich assays) by means well known in the art. Suitable detection means and methods of making them are further discussed, for  
10 example, in Tom et al., U.S. Patent No. 4,366,241, issued December 28, 1982; and in Campbell, U.S. Patent No. 4,703,017, issued October 27, 1987.

The optional solid supports for the assays can be bibulous or nonbibulous. Examples of bibulous  
15 supports include nitrocellulose, nylon, filter paper, and the like. Non-bibulous supports are ones that do not necessarily effect a chromatographic separation of the components as they advance along the solid support. Examples of such non-bibulous supports include plastic  
20 materials such as high density polyethylene, polystyrene, polyvinyl chloride, mixtures of such high density plastics, or bibulous material rendered non-bibulous by the application of non-blocking materials, such as detergents and proteins. These and other supports and  
25 methods of use are known in the art.

The invention also provides diagnostic kits comprising the L-2 specific antibodies. In addition, such kits can contain amounts of reagents for use in assaying for an analyte. Such reagents can be substrates  
30 when enzymes are used as the detection means, as well as ancillary reagents like stabilizers, extraction reagents, buffers, wash solutions, and the like. The relative amounts of the various reagents vary widely, to provide

for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. The reagents can be provided as dry powders, usually lyophilized, including excipients, which on dissolution  
5 will provide for a reagent solution having the appropriate concentrations for performing the assay. The kit can also be contained in packaging material, such as air-tight foil, or various external containers known in the art. Such external containers can contain the  
10 device, reagents, and the instructions for use of the assays.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also included within  
15 the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

#### EXAMPLE I

This Example describes the determination of the  
20 genetic makeup of the HIV-1 provirus in L-2 cells.

In initial studies the cleavage pattern of the HIV-1 Gag polypeptide expressed in *Escherichia coli* transformed with recombinant L-2 gag containing an HIV-1 wild-type pol protease gene was found to be similar to  
25 that of parental HIV-1 (LAI) particles. Therefore, the L-2 cell provirus was sequenced at other regions of the genome. Other regions of the L-2 cell proviruses genome that were sequenced included pol protease; env gp120 (SU) and gp41 (TM); and the accessory region genes such as  
30 vif, vpu, vpr, and nef. The control was the provirus in MOLT-4 cells (ATCC) persistently infected with HIV-1

(LAI), which was designated as MO/LAI was used as a control.

The polymerase chain reaction (PCR) amplification of total cellular DNA extracted from L-2 and MO/LAI was performed as previously described (Kameoka et al., Virus Genes, 12:117-129 (1996), utilizing 30 cycles of 1 min at 94°C, 4 min at 60°C, followed by a final polymerization step for 10 min at 72°C, with a GeneAmp-XL PCR kit (Perkin-Elmer Corporation, Foster City, CA). The primers used at each region are summarized in Figure 2 (Adachi et al., J. Virol., 59:284-291 (1986). The PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The expected bands were ligated into a pBluescript II SK(-) vector (Stratagene Cloning Systems, La Jolla, CA). Nucleotide sequencing of the amplified DNA segments was carried out using ( $\gamma$ -<sup>32</sup>P)ATP-labeled T3, T7, or synthetic primers by cycle sequencing according to the protocol described for the CircumVent thermal cycle sequencing kit (New England BioLabs, Beverly, MA).

The nucleotide sequences of provirus genes in the L-2 cells were compared with those determined from MO/LAI as well as the reported HIV-1 (LAI) sequences (GenBank accession number K02013) (Wain-Hobson et al., Cell, 40:9-17 (1985), (see Figure 1). Comparison of the nucleotide sequences of provirus genes to the nucleotide sequences HIV-1, LAI and HIV-1 MO/LAI genes reveals significant mutations in the *pol* protease, *env* gp41, and *nef* genes for L-2 proviral DNA. Specifically, the protease gene had an insertion mutation of T at nucleotide position 42, resulting in the appearance of a stop codon at amino acid residue 30. This explains the protease-defective nature of L-2 particles, as evidenced

by an inability to cleave the Pr55<sup>gag</sup> precursor inside L-2 cells. For the TM envelope glycoprotein, gp41, a 1-base substitution was found at nucleotide 7781 (A to G; corresponding to nucleotide 1979 in Figure 1), resulting in an amino acid substitution at amino acid residue 660, from a lysine to an arginine. The *nef* gene also had a nonsense mutation at nucleotide 8559 (G to A; corresponding to nucleotide 170 in Figure 1), resulting in the appearance of a stop codon at amino acid residue 57, as well as other mutations set forth in Figure 3. All three of these changes in the protease, gp41, and *nef* genes were specific for L-2 proviral DNA.

In contrast, the *vpr* gene was similarly truncated for both L-2 and MO/LAI proviral DNAs owing to a 1-base substitution at nucleotide 5194 (G to A; corresponding to nucleotide 54 in Figure 1), resulting in the appearance of a stop codon at amino acid residue 18. The *vif* and *vpu* genes were identical for both L-2 and MO/LAI proviral DNAs. Also, the sequencing of *env* gp120 revealed no apparent differences between L-2 and MO/LAI although both showed V1 and V2 domains that contained deletions of either eight or two amino acids, respectively, as compared to the reported HIV-1 (LAI) sequence. Therefore it is possible that these deletions in gp120 could have occurred during the long-term maintenance of HIV-1 (LAI) in different T cell lines during the instant studies, with no apparent effect on their infectivity.

The data presented here point to the specific involvement of one or more mutation(s) in the L-2 provirus *pol* protease, *env* gp41, and/or *nef* genes as the cause for the production of defective, but highly syncytium- and apoptosis-inducing particles. In

particular, the amino acid mutation at Env gp41 accounts for the higher functional activity of protease-defective particles. Furthermore, the gp120 polypeptide deletions of 8 and 2 amino acids corresponding to SEQ ID NOS:18 and 24 (Figure 1), respectively, are critical in defining the pathogenicity of L-2 particles in the context of the L-2 Nef, protease, gp41, or Vpr mutant-derived polypeptides. These L-2 particles are responsible for causing the highly pathogenic end-stages of HIV-1 disease leading to ARC and AIDS. Thus, it is important to stimulate the immune system to attack any defective L-2 particles that accumulate during infection. The use of inactivated L-2 particles, optionally in conjunction with other HIV-1 or HIV-2 immunogens, or both, nucleic acid sequences and fragments, polypeptides and fragments thereof, and antibodies, as described in this invention, alone or in combination thereof, will remove potentially harmful defective L-2 particle from HIV-1 infected tissues of individuals, leading to an improved outcome.

These results show that HIV-1 provirus from L-2 cells have mutations in the pol protease, nef, env and vpr genes that encode the pol protease, Nef, gp41, gp120 and Vpr polypeptides and result in mutations in these polypeptides.

## EXAMPLE II

### Production of Non-infectious, L-2 Particles or Modified Forms Thereof

This Example describes the production of non-infectious L-2 particles or modified forms thereof. The method of production of non-infectious, L-2 particles or modified forms thereof has essentially been described previously with some modifications (Prior et al., (1995);

Prior et al. (1996)). The modifications are related to the cell culture and downstream recovery conditions to improve both production levels and purification of virus from the growth medium containing 5-10% fetal bovine serum.

The production method for L-2 particles will mimic those used for HIV-1 described below. The initial production steps involve anion exchange chromatography. Briefly, the cell line used is the L-2 cell line. After multiple cell culture expansions with cell densities in the range of one to two million cells per milliliter, the cellular debris is separated from virus by dead-end filtration using a 1.2  $\mu$ M cellulose ester membrane. The filtered supernatant containing L-2 particles is inactivated by addition of  $\beta$ -propiolactone (1:2000 v/v), concentrated by tangential flow ultrafiltration using a 300,000 molecular weight (MW) cut-off polysulphone membrane and the concentrates are stored at  $-70^{\circ}\text{C}$ . This hold step permits analysis of in-process material in an infectivity assay to determine that infectious virus is nondetectable before further processing. Thawed concentrates are pooled, filtered and applied to TMAE Fractogel (Merck, Darmstadt, Germany), washed with 0.5M NaCl at pH 6.5 and eluted in 1.0M NaCl at pH 6.5. After immediate dilution to reduce NaCl concentration to about 0.15M, product is applied to Q-Sepharose (Pharmacia, Inc., Piscataway, NJ, USA), washed in 0.7M NaCl and eluted in 1.0M NaCl, all at pH 6.5. Following concentration/diafiltration (polysulphone, 3000 MW cut-off), product is centrifuged and the pellet is resuspended, then frozen and subjected to  $\gamma$  irradiation on dry ice. The combined inactivation steps,  $\beta$ -propiolactone and  $\gamma$  irradiation, can be validated to attain a  $> 17$  log viral inactivation capability.

Briefly, the pooled filtrate containing product is inactivated with  $\beta$ -propiolactone ( $\beta$ PL) and subjected to ultrafiltration. Briefly, following pH adjustment of the pooled filtrate to  $7.3 \pm 0.1$ ,  $\beta$ PL is added while  
5 stirring to a final concentration of 1:2,000 (v/v). After stirring for one hour, the preparation is transferred to a second mixing vessel to ensure complete viral inactivation and incubated at  $4^{\circ}\text{C}$  for 18-24 hours with continuous stirring. The temperature of the  
10 filtrate is raised to  $37^{\circ}\text{C}$  and maintained at that temperature for approximately five hours to hydrolyze residual  $\beta$ PL into an isomer of lactate and betapropionic acid derivatives. Approximately 10-200 liters of post  $\beta$ PL-treated filtrate is concentrated to 2.5-3.5 liters  
15 using a polysulfone 300,000 molecular weight (MW) cutoff membrane and diafiltered against 10-20 volumes of phosphate buffered saline. This step removes more than 95% of the growth medium-derived polypeptide from the permeate, including approximately 50% of the starting p24  
20 antigen as measured by ELISA. In this MW fraction (less than 300 kDa), p24 antigen is not associated with intact particles. The MW cutoff properties of the membrane achieves almost total retention of intact particles. The concentrate is stored frozen at  $-70^{\circ}\text{C}$ . This is a  
25 critical hold step because individual concentrates, derived from a single expansion of cell culture, can be thawed and pooled to the required lot size for subsequent chromatography. This hold step can be used for quality control to determine which concentrates to release for  
30 further processing, after conducting sterility testing and after verifying that a cell culture is free of adventitious virus and mycoplasma. This in-process check before pooling can prevent compromising large lot sizes, thus avoiding economic risk while maintaining the safety  
35 and integrity of a process.

Validating the use of  $\beta$ PL for inactivating HIV-1 is another critical safety measure. To determine the effectiveness of inactivating HIV-1 with  $\beta$ PL, a high concentrate of infectious virus is used at 0 time (starting time, pre- $\beta$ PL).  $\beta$ PL is added (1:2,000 v/v), and the decay in infectivity and  $\beta$ PL is evaluated. After adding  $\beta$ PL, samples are taken hourly,  $\beta$ PL activity is immediately neutralized with sodium sulfite, and the material is maintained at  $-70^{\circ}\text{C}$  pending analysis of the infectivity and  $\beta$ PL assays. Evaluation of inactivation is computed by subtracting the final log concentration at the time point measured from the log concentration of infectious HIV-1 at 0 time (pre- $\beta$ PL). Previous unpublished work has shown that, within 12 hours, infectious virus is either nondetectable or at trace levels, and absolute inactivation is confirmed 18 hours after  $\beta$ PL is added. Because the half-life of  $\beta$ PL in production medium is approximately 19 hours, sufficient residual  $\beta$ PL exists to continue inactivation beyond the 12-18 hours during which infectious virus is nondetectable. Therefore, to evaluate the inactivation potential for the entire production time of  $\leq 53$  hours, a fresh concentration of infectious HIV-1 is added in the form of an infectious spike to the  $\beta$ PL-treated material after 12 hours to determine the cumulative inactivation. Separate validation runs can be performed in duplicate.

For large scale cell culture and harvest of L-2 particles, the cell line used is L-2 cells. Briefly, frozen vials of L-2 cells are thawed and cultures are initiated. The culture medium throughout most of the expansion process is 10% fetal bovine serum (FBS) in RPMI-1640 with 25 mM HEPES - N-(2-hydroxyethyl)piperazine-N'-2-ethanesulphonic acid. As the cells divide, the culture volume is expanded to

larger vessels and sparged daily with 5% CO<sub>2</sub>/air. In the final expansion (from approximately 72L to a final volume of 144 L), the FBS content is reduced to about 7.5% with 5.0% FBS in RPMI-1640 and 25mM HEPES. Virus is separated  
5 from intact cells and cellular debris using a 1.2- $\mu$ m cellulose ester filter. The pooled filtrate-containing product is cooled and maintained at 4°C. The entire cell culture production cycle, from the initial thaw of cells to the harvest of the final expansion takes approximately  
10 35-45 days.

For cobalt irradiation and final formulation, the frozen, postchromatography product is removed from a biosafety level 3 (BSL-3) containment facility and on dry ice is subjected to 4.5-5.5 Mrad of cobalt irradiation.  
15 The objective is to optimize virus inactivation potential and minimize alteration to polypeptide structure to produce an immunogen. Cobalt irradiation inactivates virus by shearing nucleic acid into pieces of approximately 200 base pairs (bp), as determined by  
20 polymerase chain reaction (PCR), rendering virus biologically dysfunctional. The HIV-1 inactivation efficiency of cobalt irradiation is evaluated by aliquoting a given preparation of starting high-tier infectious virus and subjecting each sample maintained at  
25 -70°C to a single dose level of irradiation. Individual aliquots are subjected to 1.0, 2.0, 3.0, 4.0, 5.0 Mrad and measured for levels of infectivity. The study is performed in triplicate using three separate lots of infectious virus. The log reduction of infectivity is  
30 determined in primary peripheral blood mononuclear cells using the starting infectious titer following 5 Mrads of irradiation. The mean reduction in infectivity is expected to be 7 logs.

Cobalt irradiation also functions to sterilize the product. This is important because, unlike polypeptide solutions, purified HIV-1 particles such as non-infectious, L-2 particles or modified forms thereof cannot be subjected to sterile filtration techniques. A 0.2  $\mu$ m filter retains HIV-1 particles. Following irradiation, product is thawed, diluted with saline to a required dose, and emulsified with an equal volume of incomplete Freund's adjuvant. Syringes are filled for intramuscular administration with final bulk product - an off-white, viscous emulsion. Consistent sterility of product is maintained by performing formulation steps in an aseptic area supplied with high quality air such as class 100 filtered air maintained under positive pressure.

Inactivated purified product is thawed, polypeptide content determined, diluted in saline and emulsified with an equal volume of incomplete Freund's adjuvant (IFA) (Seppic Inc. Paris, France), to achieve a water:oil ratio of 1:1. All liquids and containers are maintained at 2-8°C to attain an optimal viscosity. The bulk product is filled into syringes for intramuscular administration.

To determine the purity of the final viral particle product, analytical gel-filtration chromatography is used. Analytical gel-filtration chromatography CL6B resin (Pharmacia Inc.) is packed into a 16 mm x 310 mm low-pressure Pharmacia XK16 column. The total column volume is about 60 ml. Low-pressure chromatography conditions are employed during the separation procedure to ensure that the virion remains intact, including use of a non-denaturing buffer system. The running buffer is 0.1M Tris and 0.5M NaCl, pH 8.0.

This ionic strength eliminates any adsorption of the virion onto the resin. The CL6B resin is highly crosslinked and has a MW fractionation range between 10 and 4000 kDa (molecules with a MW>4000 kDa are eluted in  
5 the void volume). The column is controlled by a Perspective Biosystems BioCAD 60 control system (Cambridge, MA. USA) at a flow rate of 2 ml min. Chromatographic profiles are monitored at two wavelengths: 230 and 280 nm. To determine HIV-1 antigen  
10 recovery and calculate mass balance, approximately 1.5 ml fractions are collected.

The non-infectious, L-2 particles or modified forms thereof produced by the procedure described above can be used for the prevention or treatment of diseases  
15 caused by HIV infection.

Throughout this application various publications have been referenced within parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in this application  
20 in order to more fully describe the state of the art to which this invention pertains.

Although the invention has been described with reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific  
25 experiments detailed are only illustrative of the invention. It should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A substantially pure nucleic acid molecule or fragment thereof that encodes a truncated Nef protein comprising a nucleic acid sequence that encodes  
5 substantially the same sequence of amino acid residues 1 through 56 of an HIV-1 Nef protein.

2. The nucleic acid molecule of claim 1 wherein said nucleic acid sequence is selected from the group consisting of:

10 a nucleic acid molecule that encodes substantially the same amino acid sequence set forth in SEQ ID NO:46; and

15 a nucleic acid molecule that encodes substantially the same sequence of amino acid residues 1 through 56 of an HIV-1 Nef peptide wherein amino acid residue 54 is aspartic acid.

3. The nucleic acid molecule of claim 2 wherein said nucleic acid sequence is selected from the group consisting of:

20 (a) nucleotides 1 through 168 of SEQ ID NO:45; and

(b) nucleotides 1 through 168 of an HIV-1 Nef peptide wherein nucleotide 161 is adenine.

4. A substantially pure fragment of the nucleic acid molecule of claim 3 comprising a nucleic acid sequence that has one or more of the following amino acid residues of SEQ ID NO:45: guanine at nucleotide 43,  
5 adenine at nucleotide 56, thymine at nucleotide 98, adenine at nucleotide 111, and adenine at nucleotide 161 and can be used to specifically detect the nucleic acid molecule of claim 3(a).

5. A substantially pure mutated HIV-1 Nef  
10 protein fragment, comprising a peptide encoded by the nucleic acid molecule fragment of claim 4.

6. An antibody or fragment thereof that specifically binds to the protein fragment of claim 5.

7. A substantially pure truncated Nef protein  
15 or fragment thereof comprising substantially the same sequence of amino acid residues 1 through 56 of an HIV-1 Nef protein.

8. The truncated Nef protein of claim 7  
wherein said sequence is selected from the group  
20 consisting of:

substantially the same amino acid sequence set forth in SEQ ID NO:46; and

substantially the same sequence of amino acid residues 1 through 56 of an HIV-1 Nef  
25 protein wherein amino acid residue 54 is aspartic acid.

9. The truncated protein of claim 8 wherein said amino acid sequence is selected from the group consisting of:

5 amino acid residues 1 through 56 of SEQ ID NO:46; and

amino acid residues 1 through 56 of an HIV-1 Nef peptide wherein amino acid residue 54 is aspartic acid.

10. An antibody or fragment thereof that  
10 specifically binds to the mutated protein of claim 7, 8 or 9.

11. A substantially pure nucleic acid molecule or fragment thereof that encodes a mutated HIV-1 Env gp41 protein containing an arginine at amino acid residue 660.

15 12. The nucleic acid molecule of claim 11 wherein nucleotide 1979 is guanine.

13. A substantially pure fragment of the nucleic acid molecule of claim 12 comprising a portion of said nucleic acid molecule that contains said guanine at  
20 nucleotide 1979 and can be used to specifically detect the nucleic acid molecule of claim 12.

14. A substantially pure mutated HIV-1 Env gp41 protein fragment, comprising a peptide encoded by the nucleic acid molecule fragment of claim 13.

25 15. An antibody or fragment thereof that specifically binds to the protein fragment of claim 14.

16. A substantially pure mutated HIV-1 *Env* gp41 protein or fragment thereof comprising an HIV-1 *Env* gp41 protein containing an arginine at amino acid residue 660.

5 17. An antibody or fragment thereof that specifically binds to the protein of claim 16.

18. A substantially pure nucleic acid molecule or fragment thereof that encodes a mutated HIV-1 *Env* gp120 protein selected from the group consisting of:

10 (a) a nucleic acid sequence that encodes an HIV-1 *Env* gp120 protein wherein:

amino acid residue 143 is either serine or arginine;

15 up to eight amino acids at amino acid residues 144 through 151 in the V1 domain are deleted; and

amino acid residue 153 is either methionine or isoleucine; and

20 (b) a nucleic acid sequence that encodes an HIV-1 *Env* gp120 protein wherein:

amino acid residue 187 is either isoleucine or valine; and

25 one or two amino acids at amino acid residues 192 and 193 in the V2 domain are deleted.

19. A substantially pure fragment of the nucleic acid molecule of claim 18 comprising a portion of the nucleic acid molecule that contains said deletions and that can be used to specifically detect the nucleic acid molecule of claim 18.

20. A substantially pure mutated HIV-1 *Env* gp120 protein fragment, comprising a peptide encoded by the nucleic acid molecule fragment of claim 19.

21. An antibody or fragment thereof that specifically binds to the protein fragment of claim 20.

22. A substantially pure mutated HIV-1 *Env* gp120 protein or fragment thereof selected from the group consisting of:

(a) the amino acid sequence of an HIV-1 *Env* gp120 protein wherein amino acid residue 143 is either serine or arginine; up to eight amino acids at amino acid residues 144 through 151 in the V1 domain are deleted; and amino acid residue 153 is either methionine or isoleucine; and

(b) the amino acid sequence of an HIV-1 *Env* gp120 protein wherein amino acid residue 187 is either isoleucine or valine; and one or two amino acids at amino acid residues 192 and 193 in the V2 domain are deleted.

23. An antibody or fragment thereof that specifically binds to the protein of claim 22.

24. A substantially pure nucleic acid molecule or fragment thereof that encodes a truncated HIV-1 *Vpr* protein comprising a nucleic acid sequence encoding substantially the same amino acid residues 1 through 17  
5 of an HIV-1 *Vpr* protein.

25. The nucleic acid molecule of claim 24 wherein said nucleic acid sequence comprises nucleotides 1 through 51 of an HIV-1 *vpr* gene.

26. A substantially pure truncated HIV-1 *Vpr*  
10 protein or fragment thereof comprising substantially the same amino acid residues 1 through 17 of an HIV-1 *Vpr* protein.

27. An antibody or fragment thereof that specifically binds to the protein of claim 26.

15 28. A substantially pure nucleic acid molecule or fragment thereof that encodes a mutated and truncated HIV-1 *Pol* protease protein comprising a nucleic acid sequence encoding substantially the same sequence of amino acid residues 1 through 13 of a wild-type HIV-1 *Pol*  
20 protease protein and amino acid residues 14 though 29 of SEQ ID NO:6.

29. The nucleic acid molecule of claim 26 wherein said nucleic acid sequence comprises nucleotides 1 through 41 of a wild-type HIV-1 *Pol* protease gene and  
25 nucleotides 42 though 87 of SEQ ID NO:5.

30. A substantially pure fragment of the nucleic acid molecule of claim 29, comprising a fragment of SEQ ID NO:5 at least 8 nucleotides in length and that can be used to specifically detect the nucleic acid molecule of claim 29.

31. A substantially pure mutated and truncated HIV-1 Pol protease protein or fragment thereof comprising substantially the same sequence of amino acid residues 1 through 13 of a wild-type HIV-1 Pol protease protein and amino acid residues 14 through 29 of SEQ ID NO:6.

32. An immunogen comprising an inactivated protease-defective viral HIV-1 particle containing one or more of the following substantially pure proteins:

an HIV-1 Env gp120 protein comprising substantially the same protein of claim 22;

a mutated HIV-1 Pol protease molecule comprising substantially the same sequence of amino acid residues 1 through 13 of a wild-type HIV-1 Pol protease protein and amino acid residues 14 through 29 of SEQ ID NO:6;

a truncated HIV-1 Nef protein or fragment thereof comprising substantially the same sequence of amino acid residues 1 through 56 of an HIV-1 Nef protein;

a mutated HIV-1 Nef protein comprising substantially the same amino acid sequence set forth in SEQ ID NO:46 or a fragment thereof comprising one or more of the following amino acid residues of SEQ ID NO:46: alanine at amino

55

acid residue 15, lysine at amino acid residue 19, valine at amino acid residue 33, and aspartic acid at amino acid residue 54;

5

a truncated HIV-1 *Nef* protein comprising substantially the same sequence of amino acid residues 1 through 56 of a *Nef* peptide wherein amino acid residue 54 is aspartic acid;

10

a truncated HIV-1 *Vpr* protein or fragment thereof comprising substantially the same amino acid residues 1 through 17 of an HIV-1 *Vpr* protein; or

15

an HIV-1 *Env* gp41 protein or fragment thereof comprising substantially the same amino acid sequence of an HIV-1 *Env* gp41 protein containing an arginine at amino acid residue 660.

33. The immunogen of claim 32 further comprising one or more suitable adjuvants.

20 34. A method of detecting a mutated HIV-1 gene selected from the group consisting of a *nef* gene, an *env* gp41 gene and an *env* gp120 gene or fragments thereof in a sample of cells, lysed cells or extracellular fluid from an individual, comprising the steps of:

25

obtaining from an individual a sample of cells, lysed cells or extracellular fluid suspected of containing said mutated gene or fragments thereof;

56

contacting said sample with a measured amount of the nucleic acid molecule of claims 4, 13 or 19, respectively, to hybridize with said gene or fragment thereof; and

5

determining the presence of said hybridized molecule to detect the presence of the mutated gene or fragments thereof in said sample.

35. A method of detecting the presence of a mutated HIV-1 protein selected from the group consisting of a *Nef* protein, an *Env* gp41 protein and an *Env* gp120 protein or fragments thereof in an individual, comprising the steps of:

15

obtaining from an individual a sample of cells, lysed cells or extracellular fluid suspected of containing a mutated HIV-1 protein or fragments thereof;

20

contacting said sample with a measured amount of the antibody of claims 6 or 10, 15 or 17, or 21 or 23, respectively, to form a complex of the antibody and said protein or fragment thereof; and

25

determining the presence of said complex to detect the presence of the mutated protein or fragments thereof in said sample.

36. A method of reducing the severity of HIV-1 infections in individuals, comprising contacting protease-defective viral particles containing one or more mutated HIV-1 Nef proteins, mutated HIV-1 Env gp41 proteins, or mutated HIV-1 Env gp120 proteins or fragments thereof present on cells, in lysed cells or in extracellular fluid with an effective amount of the antibody of claims 6 or 10, 15 or 17, or 21 or 23, respectively, to inhibit the activity of said proteins or fragments thereof.

37. The method of claim 36 wherein said antibody is attached to a moiety selected from the group consisting of radioactive moieties, chemotherapeutic moieties and chemotoxic moieties.

38. A method of reducing or preventing apoptotic cell lysis in an HIV-1 seropositive or seronegative individual, comprising administering an immunologically effective amount of the immunogen of claim 32 or 33 to the individual.

FIGURE 1

pol prot. LAI	nucleotide		
	40	AA-GATAGGGGGCAACTAAAGGAAGCTCTATTAGATACAGGAGCAGATGAT	
	MO/LAI	K I G G Q L K E A L L D T G A D D	SEQ. ID NO. 1
	L-2	T.....	SEQ. ID NO. 2
VPR	nucleotide		
	1	ATGGAACAGCCCAAGACCAAGAGCCACAGAGGGAGCCACCAATGAATGGACACTA	
	MO/LAI	M E Q A P E D Q G P Q R E P H N E W T L	SEQ. ID NO. 7
	L-2	.....A.....	SEQ. ID NO. 8
env gp120 LAI	nucleotide		
	424	AGTAGTATACCAATAGTAGTAGCGGGGAATGATG	
	MO/LAI	S S N T N S S S G E M M	SEQ. ID NO. 9
	L-2	R # # # # # # # # # # # # # # # #	SEQ. ID NO. 10
env gp120 LAI	nucleotide		
	424	AGTAGTATACCAATAGTAGTAGCGGGGAATGATG	
	MO/LAI	S S N T N S S S G E M M	SEQ. ID NO. 11
	L-2	R # # # # # # # # # # # # # # # #	SEQ. ID NO. 12
env gp120 LAI	nucleotide		
	424	AGTAGTATACCAATAGTAGTAGCGGGGAATGATG	
	MO/LAI	S S N T N S S S G E M M	SEQ. ID NO. 13
	L-2	R # # # # # # # # # # # # # # # #	SEQ. ID NO. 14
env gp120 LAI	nucleotide		
	424	AGTAGTATACCAATAGTAGTAGCGGGGAATGATG	
	MO/LAI	S S N T N S S S G E M M	SEQ. ID NO. 15
	L-2	R # # # # # # # # # # # # # # # #	SEQ. ID NO. 16
env gp120 LAI	nucleotide		
	424	AGTAGTATACCAATAGTAGTAGCGGGGAATGATG	
	MO/LAI	S S N T N S S S G E M M	SEQ. ID NO. 17
	L-2	R # # # # # # # # # # # # # # # #	SEQ. ID NO. 18
env gp120 LAI	nucleotide		
	424	AGTAGTATACCAATAGTAGTAGCGGGGAATGATG	
	MO/LAI	S S N T N S S S G E M M	SEQ. ID NO. 19
	L-2	R # # # # # # # # # # # # # # # #	SEQ. ID NO. 20
env gp120 LAI	nucleotide		
	424	AGTAGTATACCAATAGTAGTAGCGGGGAATGATG	
	MO/LAI	S S N T N S S S G E M M	SEQ. ID NO. 21
	L-2	R # # # # # # # # # # # # # # # #	SEQ. ID NO. 22
env gp120 LAI	nucleotide		
	424	AGTAGTATACCAATAGTAGTAGCGGGGAATGATG	
	MO/LAI	S S N T N S S S G E M M	SEQ. ID NO. 23
	L-2	R # # # # # # # # # # # # # # # #	SEQ. ID NO. 24

## FIGURE 1 CONT.

env gp41	LAI	nucleotide	1951	1989	
			ATTGAAGAAATCGCAAAACCCAGCAAGAAAAGAAATGAACAA	SEQ. ID NO. 25	
		I E E S Q N Q Q E K N E Q	SEQ. ID NO. 26		
		MO/LAI		SEQ. ID NO. 27	
				SEQ. ID NO. 28	
L-2			SEQ. ID NO. 29		
			SEQ. ID NO. 30		
	651	663			
		amino-acid residue			

nef	LAI	nucleotide	130	180	
			ACAAGTAGCAATACAGCAGCTACCAATGCTGTGCTGGCTAGAGCA	SEQ. ID NO. 31	
		T S S N T A A T N A A C A W L E A	SEQ. ID NO. 32		
		MO/LAI		SEQ. ID NO. 33	
				SEQ. ID NO. 34	
L-2			SEQ. ID NO. 35		
			SEQ. ID NO. 36		
	44	57			
		amino acid residue			

FIGURE 2

PRIMERS USED FOR PCR AMPLIFICATION OF HIV-1 PROVIRUS IN L-2						
Primer		Nucleotide No.	Sequence	Target gene(s)	Amplified size (bp)	SEQ ID NO.
gag-d	Sense	(1921-1942)	5' CAGAAAGGCAATTTAGGAACC 3'			
JA-20	Antisense	(2590-2571)	5' CCTGGCTTTAATTTTACTGG 3'			37
				pol (protease)	670	38
pol-3	Sense	(4808-4826)	5' GTACAGGGGAAAGAATA 3'			
env-3	Antisense	(6341-6317)	5' CCCATAATAGACTGTGACCCACAA 3'			39
				vif, vpr, vpu	1538	40
EN-D-Sal	Sense	(6221-6238)	5' TGAGTCGACATGAGAGTGAAGGAGAAATAT 3'			
EA-Pst	Antisense	(8782-8763)	5' TCCTGCAGCTTATAGCAAAATCCTTTCCA 3'			41
				env (gp120, gp41)	2562	42
EN53	Sense	(8655-8678)	5' TCAATGCCACAGCCATAGCAGTAG 3'			
LR3	Antisense	(9508-9568)	5' CAGTGGGTTCCCTAGTTAGCC 3'			43
				nef	934	44

## FIGURE 3

SEQ. ID NO. 45

1	11	21	31	41
ATGGGTGGCA	AGTGGTCAAA	AAGTAGTGTG	GTTGGATGGC	CTGCTGTAAG
51	61	71	81	91
GGAAT A AATG	AGCGAGCTG	AGACAGCAGC	AGATGGGGTG	GGAGCAG T AT
101	111	121	131	141
CTCGAGACCT	A GAAAA CAT	GGAGCAATCA	CAAGTAGCAA	TACAGCAGCT
151	161	171		
ACCAATGCTG	A TTGTGCCT A	GCTAGAAGCA		
181	191	201		
CAAGAGGAGG	AGGAGGTGGG	TT - - -		

**FIGURE 4****SEQ. ID NO. 46**

(amino) - NH<sub>2</sub> - MGCKWSKSSV VGWP **A** VRE **K** MRRRAEPAADGV GA **V** SRDLEKH GAIT  
SSNTAA TNA **D** CA - COOH (carboxy)

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/06690**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/139.1, 148.1, 160.1, 188.1, 205.1, 208.1; 435/5, 6, 236, 237; 436/504; 530/387.9, 388.35, 389.4, 395, 403; 536/23.72.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	KAMEOKA et al. Induction of apoptosis by protease-defective particle preparations of human immunodeficiency virus type 1 is specific to a subset of U937-derived subclones. International Immunology. 1996. Vol. 8, No. 11, pages 1687-1697, see entire document.	32 ----- 1-31, 33-38
X, P ----- Y, P	BAHMANI et al. Production of Doughnut-shaped, protease-defective particles from a human T cell clone carrying a provirus with specific mutations in the env, pol, vpr, and nef genes. AIDS Research and Human Retroviruses. 1997. Vol. 13, No. 6, pages 523-526, see entire document.	1-5, 7-9, 11-14, 16, 18-20, 22, 24- 26, 28-32 ----- 6, 10, 15, 17, 21, 23, 27, 33-38

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/06690

A. CLASSIFICATION OF SUBJECT MATTER:  
IPC (6):

A61K 39/21, 39/42; C07H 21/02, 21/04; C07K 14/155, 16/10; C12N 7/04, 7/08; C12Q 1/68, 1/70; G01N 33/577, 33/60, 33/68.

A. CLASSIFICATION OF SUBJECT MATTER:  
US CL :

424/139.1, 148.1, 160.1, 188.1, 205.1, 208.1; 435/5, 6, 236, 237; 436/504; 530/387.9, 388.35, 389.4, 395, 403; 536/23.72.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING  
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

- Group I, claim(s) 1-5, 7-9 and 34, truncated nef proteins and nucleic acids and method of use.
- Group II, claim(s) 6, 10 and 35, drawn to antibodies to mutated nef protein.
- Group III, claim(s) 11-14, 16 and 34, drawn to mutated gp41 proteins and nucleic acids and method of use.
- Group IV, claim(s) 15, 17 and 35, drawn to antibodies to mutated gp41 and method of use.
- Group V, claim(s) 18-20, 22 and 34, drawn to mutated gp120 proteins and nucleic acids and method of use.
- Group VI, claim(s) 21, 23 and 35, drawn to antibodies to mutated gp120 and method of use.
- Group VII, claim(s) 24-26, drawn to mutated vpr proteins and nucleic acids.
- Group VIII, claim(s) 27, drawn to antibodies to mutated vpr.
- Group IX, claim(s) 28-31, drawn to mutated pol proteases and nucleic acids.
- Group X, claim(s) 32-33 and 38, drawn to defective HIV-1 particles and method of use.
- Group XI, claim(s) 36-37, drawn to method of treating HIV-1 using antibodies.

The inventions listed as Groups I-XI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the products of Groups I-X are directed to different mutated proteins, antibodies and nucleic acids which differ in their physical and chemical structures such as primary sequence and immunologic activity and are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general inventive concept. Further, the methods of Groups I-VI and X-XI differ in their industrial applicability, reagents and method steps and are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general inventive concept.



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> A61K 39/21, 39/42, C07H 21/02, 21/04, C07K 14/155, 16/10, C12N 7/04, 7/08, C12Q 1/68, 1/70, G01N 33/577, 33/60, 33/68	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/44945</b>  <b>(43) International Publication Date:</b> 15 October 1998 (15.10.98)
<b>(21) International Application Number:</b> PCT/US98/06690  <b>(22) International Filing Date:</b> 3 April 1998 (03.04.98)  <b>(30) Priority Data:</b> 60/043,047 4 April 1997 (04.04.97) US  <b>(71) Applicant:</b> THE IMMUNE RESPONSE CORPORATION [US/US]; 5935 Darwin Court, Carlsbad, CA 92008 (US).  <b>(72) Inventor:</b> LUFTIG, Ronald, B.; 5120 Pike Drive, Metairie, LA 70003 (US).  <b>(74) Agents:</b> STEINHARDT, Paul, C. et al.; Campbell & Flores LLP, Suite 700, 4370 La Jolla Village Drive, San Diego, CA 92122 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> NON-INFECTIOUS, PROTEASE DEFECTIVE HIV PARTICLES AND NUCLEIC ACID MOLECULES ENCODING THEREFOR  <b>(57) Abstract</b>  This invention is directed toward mutated DNA, proteins, or protein fragments and particles from the L-2 cell line. The invention is also directed to diagnostic, prophylactic and therapeutic methods of making and using the DNA, protein and particles.		

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NON-INFECTIOUS, PROTEASE DEFECTIVE HIV PARTICLES AND NUCLEIC ACID MOLECULES ENCODING THEREFOR

This application is based on, and claims the benefit of, U.S. Provisional Application No. 60/043,047, filed April 4, 1997, and entitled NON-INFECTIOUS, PROTEASE DEFECTIVE HIV PARTICLES AND NUCLEIC ACID MOLECULES ENCODING THEREFOR.

BACKGROUND OF THE INVENTION

This invention relates to human immunodeficiency viruses in general and, more specifically to non-infectious vaccines for the treatment of human immunodeficiency disease.

AIDS is a lethal disease caused by human immunodeficiency viruses HIV-1 and HIV-2. This disease is characterized by a long, latent asymptomatic phase. During this phase the HIV particles are suppressed by the body's immune defense system. The next phase of the disease occurs when the body's immune system is no longer able to suppress the viral particles. At this stage of the disease, the HIV viral particles attack and destroy a key component of the body's immune defense system called CD4<sup>+</sup> cells. Once these cells are destroyed, the third and final phase of the disease occurs. During this last phase, the body is extremely susceptible to infections by many different kinds of diseases. These so-called secondary infections are the cause of death in many AIDS patients.

One of the central mysteries about AIDS is why HIV particles can exist in the body for so long, yet the patient remains asymptomatic. As a corollary to this riddle, there is no certain explanation why the immune system suddenly fails to be effective against the particles, thus enabling the second phase of the disease.

One theory for the sudden onslaught of the HIV particles on the CD4<sup>+</sup> cells is that this onslaught is made by "defective" HIV particles. These new, defective particles are not recognized by the body's immune system.

5 These defective particles have mutations in some of the key polypeptides of an HIV viral particle, yet somehow are still able to affect sufficient damage on the CD4<sup>+</sup> cells by means other than the classical invasion of the cells.

10 Surprisingly, non-infectious HIV particles produced by a cell called "L-2" fuse more efficiently than wild-type HIV with a subpopulation of CD4<sup>+</sup> cells. The enhanced fusion is caused by the 4-fold increased level of gp120, the two deletion mutations in gp120,  
15 altered conformational epitopes of gp120 and/or a combination of these factors, as well as mutant forms of Nef, gp41, protease, and Vpr, as discussed below. The fusion is far from the typical action of normal HIV infectious viral particles, in that it leads to the  
20 stimulation and release of Il-2R, Fas ligand and interferon-gamma 1 from a small subset of CD4<sup>+</sup> cells (Kameoka et al., International Immunology 9(10):1453-1462 (1997)). This occurs after L-2 particle fusion with these CD4<sup>+</sup> cells, a high percentage of uninfected, healthy  
25 neighboring CD4<sup>+</sup>, as well as CD8<sup>+</sup> cells, are prematurely thrown into a programmed cell death cycle, ultimately resulting in apoptosis. Apoptosis is a natural process in most cells in the body, but only after such cells have become senescent. Fusion of L-2 particles with CD4<sup>+</sup> cells  
30 can also cause such cells to form multinucleated protoplasmic masses called "syncytia", which die shortly thereafter.

Thus, there exists a need to detect such defective HIV particles in the infected population; both  
35 for asymptomatic carriers, as well as for ARC (AIDS

Related Complex) or AIDS patients. Furthermore, a need exists for prophylactic, immunogenic, and therapeutic agents for such defective particles, as well as polypeptides or fragments of polypeptides derived from the particles or their mutant nucleic acid sequences. This need exists due to the fact that mutations found in L-2 particles are normally produced in HIV infected individuals, so that development of immunity to them by natural or artificial means is a key to thwarting the pathogenic process leading to ARC and AIDS. This invention provides these advantages and more.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 sets forth the mutations in the nucleotide sequences and deduced amino acid residues of the defective HIV-1 provirus in L-2 cells compared with those in MO/LAI and LAI cells. The portions of the nucleotide sequences of the relevant *pol* protease (prot.), *vpr*, *env* (gp120 and gp41), and *nef* gene regions in wild-type HIV-1 (LAI), as well as the proviruses of L-2 or MO/LAI, are shown in the upper part for each gene. The portions of the deduced amino acid sequences of Pol, Vpr, Env and Nef polypeptides setting forth the mutations are shown below the nucleotide sequences. The wild-type LAI control sequences are taken from previously reported sequences for HIV-1 (LAI), in GenBank. The sequences identical with those of wild-type LAI are indicated by dots. An asterisk (\*) indicates the appearance of stop codons. For the upper part, a dash (-) indicates a deletion in nucleotide sequences. For the amino acid sequences represented in the lower part, a dash indicates sequences identical with the wild type HIV-1 (LAI) sequence. A hatchmark (#) indicates a deletion in deduced amino acid residues. Three pluses (+) indicates any three nucleotides coding for the corresponding amino acid shown in the deduced amino acid sequence.

Figure 2 sets forth the primers used for PCR amplification of protease-defective HIV-1 provirus in L-2 cells. Nucleotide numbers are those of the HIV-1 molecular clone pNL432 (Adachi et al., J. Virol, 59:284-291 (1986)).

Figure 3 sets forth the partial nucleic acid sequence of the mutated *nef* gene of the HIV-1 protease-defective provirus in the L-2 cell, where the individual bases that differ from the wild-type LAI HIV-1 virus in GenBank are encircled.

Figure 4 sets forth the sequence of the truncated *Nef* polypeptide coded for by the mutated *nef* gene of the L-2 HIV-1 provirus. The encircled amino acids differ from the wild-type LAI HIV-1 virus amino acid sequence set forth in GenBank.

#### SUMMARY OF THE INVENTION

This invention is directed to nucleic acid molecules and fragments thereof containing mutations in certain genes of the HIV-1 virus, and the polypeptides and fragments thereof that are encoded by such mutated nucleic acid molecules, antibodies to such polypeptides, nucleic acid and antibody-based assays, as set forth below, as well as therapeutic uses of such antibodies. This invention is also directed to immunogens, and compositions of immunogens and adjuvants, containing such mutated HIV-1 viruses and in the production and use in therapeutic and prophylactic (immunogenic) methods and compositions.

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to the identification of mutations within pathologically important genes of the human immunodeficiency genome (HIV) which render viral particles non-infectious. The non-infectious phenotype of the viral particles can advantageously be used as a natural source of material for the production of immunogens for vaccination against or treatment of Acquired Immunodeficiency Syndrome (AIDS). The mutations identified within the various pathologically important genes can also be advantageously used to engineer new non-infectious viral particles for use as immunogens against essentially any type of HIV isolate.

In one embodiment, the invention is directed to the mutations discovered in various pathologically important genes of the non-infectious, protease-defective HIV-1 provirus harbored by L-2 cells. The partial sequences illustrating the nucleotide and amino acid changes caused by these mutations are set forth in Figures 1, 3 and 4.

The formation of syncytia and apoptosis of bystander T cells appear to be a mechanism of HIV-1-induced pathogenesis, leading to the diminution of T cells. (Amazon and Capon, Immunol... Today, 12:102-105 (1991); Malderelli et al., J. Virology, 69:6457-6465 (1995); and Sodroski et al., Nature, 322:470-474 (1996)). It has been reported that subclones obtained from surviving cells after MT-4, a CD4 human T cell line, had been infected with wild-type LAI or infectious molecular clone pNLA32-derived viruses, were predominantly producers of infectious but less cytopathogenic virus particles (Yunoki et al., Arch. Virol., 116:143-158 (1991); Nishino et al., Arch. Virol., 120:181-192 (1991)). The provirus in these persistently infected

cell clones carried mutations in accessory genes, such as *vif*, *vpr*, and *vpu*. (Nishino et al., J. Gen. Virol., 75:2241-2251 (1994), and Kishi et al., J. Virol., 69:7507-7518 (1995)). However, about 10% of the subclones  
5 produced above with LAI virus showed defective phenotypes even in essential gene products. In particular, one of these, named the L-2 cell clone, was a large-scale producer of protease-defective, gp120-containing noninfectious particles. (Ikuta et al., J. Cancer Res.,  
10 78:118-123 (1988)).

Surprisingly, the protease-defective viral particles produced from the L-2 cell clone rapidly induce syncytium formation of uninfected T cells by virus-to-cell fusion (fusion from without), without the need for  
15 viral replication. (Ohki et al., J. AIDS, 4:1233-1240 (1991)). In addition, the L-2 particles have a significantly higher (>fivefold) activity for apoptosis induction in primary peripheral blood T cells, as well as a certain subset of U937 cells when compared with soluble  
20 recombinant gp120 or the wild-type HIV-1 (LAI) strain (Kameoka et al., Int. Immunol., 11:1687-1697 (1996); Kameoka et al., J. Clin. Microbiol., 35:41-47 (1997)). Sodium dodecyl sulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis demonstrates that the Gag precursor  
25 polypeptide in L-2 particles was not cleaved to mature Gag polypeptides and that the Env/Gag ratio in L-2 particles was about fourfold higher than that in HIV-1 (LAI) wild-type particles produced from persistently infected MOLT-cells (Yunoki et al., Arch. Virol.,  
30 116:143-158 (1991); Kameoka et al. (1997) supra)).

"Substantially pure" when used to describe the state of the claimed nucleic acids, polypeptides or fragments thereof, is intended to mean that the claimed

molecules are free of at least a portion of the contents associated with or occurring with the claimed nucleic acids, polypeptides or fragments thereof in the native environment.

5           As used herein, the term "substantially" or "substantially the same" when used in reference to a nucleotide or amino acid sequence is intended to mean that the nucleotide or amino acid sequence shows a considerable degree, amount or extent of sequence  
10 identity when compared to a reference sequence. Such considerable degree, amount or extent of sequence identity is further considered to be significant and meaningful and therefore exhibit characteristics which are definitively recognizable or known. Thus, a  
15 nucleotide sequence which is substantially the same nucleotide or amino acid sequence as a claimed sequence, refers to a sequence which exhibits characteristics that are definitively known or recognizable as representing the claimed nucleotide or amino acid sequence and minor  
20 modifications thereof.

          In regard to substantially the same nucleotide sequences, such characteristics include for example, specific hybridization of a nucleotide sequence to a claimed nucleic acid or its complement. Hybridization  
25 principles and methods for determining hybridization specificity are well established and are known by one skilled in the art. Such principles and methods can be found in, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor  
30 Laboratory, New York (1992), and in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, MD (1989). Thus, it is not necessary that two nucleic acids exhibit sequence identity to be

substantially complimentary, only that they can specifically hybridize or be made to specifically hybridize without detectible cross reactivity with other similar sequences.

5           In regard to substantially the same amino acid sequence, such characteristics which are definitively recognizable or known include, for example, maintaining the biological function of the polypeptide or substitution of conservable amino acid residues. It is  
10 understood that limited modifications may be made without destroying the biological function of the claimed polypeptides or fragments thereof, and that only a portion of the entire primary structure can be required in order to effect activity. For example, the claimed  
15 polypeptides of the invention have an amino acid sequence substantially similar to those indicated for the L-2 polypeptides shown in Figures 1 or 4, but minor modifications of these sequences which do not destroy their activity also fall within the definition of the  
20 polypeptides claimed as such. Moreover, fragments of the sequences of the L-2 polypeptides shown in Figures 1 and 4, are similarly included within the definition. It is understood that minor modifications of primary amino acid sequence may result in polypeptides which have  
25 substantially equivalent or enhanced function as compared to the sequences of the L-2 polypeptides set forth in Figures 1 and 4. These modifications can be deliberate, as through site-directed mutagenesis, or may be accidental such as through mutation in hosts which  
30 produce the claimed polypeptides. All of these modifications are included as long as the biological functions are retained.

As used herein, the term "fragment" when used in reference to a nucleic acid encoding the claimed polypeptides is intended to mean a nucleic acid having substantially the same sequence as a portion of a nucleic acid encoding the claimed *Pol* protease, *Nef*, *Env* gp41, *Env* gp120 or *Vpr* polypeptides or fragments thereof. The nucleic acid fragment is sufficient in length and sequence to selectively hybridize to a *Pol* protease, *Nef*, *Env* gp41, gp120 or *Vpr* encoding nucleic acid or a nucleotide sequence that is complimentary to a *Pol* protease, *Nef*, *Env* gp41, gp120 or *Vpr* encoding nucleic acid. Therefore, fragment is intended to include primers for sequencing and polymerase chain reaction (PCR) as well as probes for nucleic acid blot or solution hybridization.

As used herein, the term "functional fragment" when used in reference to a *Pol* protease, *Nef*, *Env* gp41, gp120 or *Vpr* polypeptide is intended to refer to a portion of a *Pol* protease, *Nef*, *Env* gp41, gp120 or *Vpr* polypeptide which still retains some or all or the apoptotic inducing activity of the L2 particles containing one or more of these polypeptides as described herein. The term is also intended to include polypeptides that include, for example, modified forms of naturally occurring amino acids such as D-stereoisomers, non-naturally occurring amino acids, amino acid analogues and mimics so long as such polypeptides retain functional activity as defined above.

The HIV-1 provirus from L-2 cells has been analyzed with respect to genetic mutations that result in non-infectious HIV particles. Genes of HIV-1 provirus from L-2 cells have been isolated and sequenced and

compared to the wild type HIV-1 MO/LAI and LAI isolates. Various mutations have been observed in several viral genes encoding viral polypeptides, including the *nef*, *env*, *pol* and *vpr* genes.

5           One aspect of the instant invention is a substantially pure nucleic acid molecule encoding a mutant HIV-1 Nef protein, containing the encircled L-2 mutations set forth as SEQ ID NO: 45 (Fig. 3), and any fragments of such a nucleic acid molecule containing at  
10   least one of the encircled L-2 mutations which can be used to specifically detect the mutated nucleic acid or protein.

          The *nef* gene of HIV-1 provirus from L-2 cells was compared to the wild type HIV-1 in MO/LAI and LAI  
15   isolates GenBank accession No. K02013. Several mutations in the *nef* gene compared to the wild type HIV-1 *nef* gene sequence were observed. One mutation is the substitution of A for G at nucleotide 169 of the *nef* gene. This mutation results in a stop codon at amino acid residue 57  
20   such that the Nef polypeptide is truncated and contains amino acids 1 through 56.

          The invention provides a substantially pure nucleic acid molecule or fragment thereof that encodes a truncated Nef protein comprising a nucleic acid sequence  
25   that encodes substantially the same sequence of amino acid residues 1 through 56 of an HIV-1 Nef polypeptide.

          Another mutation found in the *nef* gene of HIV-1 provirus from L-2 cells is the substitution of G for the A at nucleotide 43 of the wild type *nef* gene. This  
30   mutation changes Thr at amino acid residue 15 of the Nef polypeptide to Ala. An additional mutation found in the

*nef* gene of HIV-1 provirus from L-2 cells is the substitution of A for the G at nucleotide 56 of the wild type *nef* gene. This mutation changes Arg at amino acid residue 19 of the Nef polypeptide to Lys.

5 Additional mutations of the *nef* gene of HIV-1 provirus from L-2 cells were found. One of these mutations is the substitution of T for the C at nucleotide 98 of the wild type *nef* gene. This mutation changes Ala at amino acid residue 33 of the Nef  
10 polypeptide to Val. Still another of these mutations is the substitution of A for the C at nucleotide 161 of the wild type *nef* gene. This mutation changes Ala at amino acid residue 54 of the Nef polypeptide to Asp.

An additional mutation found in the *nef* gene of  
15 HIV-1 provirus from L-2 cells is the substitution of A for the G at nucleotide 111 of the wild type *nef* gene. This mutation results in no change in the amino acid at residue 37 of the encoded Nef polypeptide since the nucleotide substitution occurs in the third position of  
20 the codon for Leu and all nucleotide substitutions at this position would encode for Leu. Therefore, nucleotide 111 of the *nef* gene can be any of the four nucleotides.

The invention also provides a substantially  
25 pure nucleic acid or fragment thereof that encodes a Nef polypeptide comprising a nucleotide sequence that encodes substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Ala at amino acid residue 15. The invention additionally provides a substantially pure  
30 nucleic acid or fragment thereof that encodes a Nef polypeptide comprising a nucleotide sequence that encodes

substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Lys at amino acid residue 19.

The invention further provides a substantially pure nucleic acid or fragment thereof that encodes a Nef polypeptide comprising a nucleotide sequence that encodes substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Val at amino acid residue 33. The invention also provides a substantially pure nucleic acid or fragment thereof that encodes a Nef polypeptide comprising a nucleotide sequence that encodes substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Asp at amino acid residue 54.

The nucleotide substitutions and amino acid mutations found in the *nef* gene of HIV-1 provirus from L-2 cells and the encoded Nef polypeptide are shown in Figures 3 and 4, respectively.

A second aspect of the instant invention is a substantially pure nucleic acid molecule encoding a mutated HIV-1 *env* gp41 protein containing the L-2 mutation set forth in SEQ ID NO: 29 (Fig. 1) and any fragment of such nucleic acid molecule containing the mutation which can be used to specifically detect the mutated nucleic acid or protein.

A mutation in the L-2 cell derived HIV-1 provirus *env* gene encoding the Env gp41 polypeptide has also been identified. The mutation is the substitution of G for the A at nucleotide 7781 (nucleotide 1979 in Figure 1) in a wild type *env* gene. This mutation changes Lys at amino acid residue 660 of the Env gp41 polypeptide to Arg.

The invention also provides a substantially pure nucleic acid or fragment thereof that encodes an Env gp41 polypeptide comprising a nucleotide sequence that encodes substantially the same amino acid sequence of an HIV-1 Env gp41 polypeptide containing Arg at amino acid residue 660.

A third aspect of the instant invention is a substantially pure nucleic acid molecule encoding a mutated HIV-1 pol protease protein containing the L-2 mutation set forth in SEQ ID NO:5 (Fig. 1) and any fragment of such nucleic acid molecule containing the mutation which can be used to specifically detect the mutated nucleic acid or protein.

A mutation in the L-2 cell derived HIV-1 provirus pol protease gene encoding the pol protease polypeptide has also been identified. The mutation is the insertion of T after the A at nucleotide 41 in wild type pol protease gene. This mutation results in a frameshift and the appearance of a stop codon at amino acid residue 30 of the Pol protease polypeptide. This results in a 29 amino acid Pol protease polypeptide with the carboxyl-terminal amino acid sequence AsnAspArgGlyAlaThrLysGlySerSerIleArgTyrArgSerArg (SEQ ID NO:6) starting at amino acid residue 14.

The invention additionally provides a substantially pure nucleic acid or fragment thereof that encodes a Pol protease polypeptide comprising a nucleotide sequence that encodes substantially the same sequence of amino acid residues 1 through 13 of an HIV-1 Pol protease polypeptide fused at the carboxyl-terminus to substantially the same amino acid sequence

AsnAspArgGlyAlaThrLysGlySerSerIleArgTyrArgSerArg (SEQ ID NO:6).

A fourth aspect of the invention is directed to a substantially pure nucleic acid molecule encoding a mutant HIV-1 gp120 protein containing the L-2 mutations set forth in SEQ ID NOS:17 and 23 (Fig. 1), and any fragment of said nucleic acid containing one or more of the L-2 mutations which can be used to specifically detect the mutated nucleic acid or protein.

10 Mutations in the L-2 cell derived HIV-1 provirus env gene encoding the Env gp120 polypeptide have also been identified. A mutant Env gp120 found in HIV-1 provirus from L-2 cells has a substitution of Arg for the Ser at amino acid residue 143, substitution of Ile for  
15 the Met at position 153 and deletion of eight amino acids, from 144 through 151, of the wild type HIV-1 from LAI cells as well as substitution of Val for the Ile at amino acid residue 187 and deletion of two amino acids, 192 and 193, of the wild type HIV-1 from LAI cells. The  
20 substitution of Arg at amino acid position 143 occurred due to nucleotide substitutions that changed the codon for Ser to the codon for Arg. Any mutation that results in changing the nucleotides encoding Ser 143 (nucleotides 427 through 429) to Arg can be used in the nucleic acids  
25 of the invention. For example, nucleotides 427 through 429, AGT, can be substituted with nucleotides that result in a codon for Arg, such as substitution of A for the T at nucleotide 429, substitution of G for the T at nucleotide 429, substitution of C for the A at nucleotide  
30 427, substitution of C for the A at position 427 combined with substitution of C for the T at nucleotide 429, substitution of C for the A at position 427 combined with the substitution of A for the T at nucleotide 429, and

substitution of C for the A at position 427 combined with the substitution of G for the T at nucleotide 429, all of which result in substitution of Arg for Ser at amino acid position 143. The substitution of Ile for Met at amino acid residue 153 results from substitution of A for the G at nucleotide 459. The substitution of Val for Ile at amino acid residue 187 results from substitution of G for A at nucleotide 559. This mutant Env gp120 mutant is identical to a Env gp120 mutant found in HIV-1 from MO/LAI (see Figure 1).

The invention also provides a substantially pure nucleic acid or fragment thereof that encodes an Env gp120 polypeptide comprising a nucleotide sequence that encodes substantially the same amino acid sequence of an HIV-1 Env gp120 polypeptide containing Arg at amino acid residue 143, Ile at amino acid residue 153, and Val at the amino acid residue corresponding to amino acid residue 187 of a wild type HIV-1 Env gp120 polypeptide and absent amino acid residues corresponding to amino acid residues 144 through 151 and 192 through 193 of a wild type HIV-1 Env gp120 polypeptide.

A fifth aspect of the invention is directed to a substantially pure nucleic acid molecule encoding a mutant HIV-1 Vpr protein containing the L-2 mutation set forth in SEQ ID NO: 11 (Fig. 1), and any fragment of such a nucleic acid molecule containing the L-2 mutation which can be used to specifically detect the mutated nucleic acid or protein.

A mutation in the L-2 cell derived HIV-1 provirus vpr gene encoding the Vpr polypeptide has also been identified. The mutation is the substitution of A for the G at nucleotide 5194 (nucleotide 54 in Figure 1).

in a wild type *vpr* gene. This mutation introduces a stop codon at amino acid residue 18 such that the *vpr* gene encodes a truncated Vpr polypeptide containing amino acid residues 1 through 17.

5           The invention also provides a substantially pure nucleic acid or fragment thereof that encodes a Vpr polypeptide comprising a nucleotide sequence that encodes substantially the same sequence of amino acid residues 1 through 17 of an HIV-1 Vpr polypeptide.

10           In addition to the nucleic acids described above encoding individual Nef, Env gp41, Pol protease, Env gp120 and Vpr polypeptide mutants, the invention also provides substantially purified nucleic acids encoding combinations of these mutant polypeptides. In one  
15           embodiment, any of the mutations in an individual polypeptide can be used alone or in combination with one or more additional mutations in the same polypeptide. Thus, the invention provides a substantially pure nucleic acid or fragment thereof that encodes a HIV-1 polypeptide  
20           having one or more of the mutations described above.

          For example, a nucleic acid encoding a Nef polypeptide can include the deletion mutant, which encodes amino acid residues 1 through 56 of the Nef polypeptide, and any combination of one or more of the  
25           amino acid substitutions. The invention provides a nucleic acid encoding the truncated Nef polypeptide containing amino acid residues 1 through 56 and Ala at amino acid residue 15. A nucleic acid can also encode the truncated Nef polypeptide with Lys at amino acid  
30           residue 19. Similarly, any of the amino acid substitution mutations in the Nef polypeptide described above can be combined with the truncated Nef polypeptide.

Alternatively, any of the amino acid substitution mutations can be combined with each other in the full length Nef polypeptide or a fragment thereof.

The invention thus provides a substantially  
5 pure nucleic acid or fragment thereof that encodes at least one of the mutations described above and can, where more than one mutation has been described in an individual polypeptide, contain any combination of two or more mutations, including as many as all of the mutations  
10 described for an individual polypeptide. An example of such a nucleic acid encoding an individual polypeptide having multiple mutations is shown in Figure 3 (SEQ ID NO:45).

In another example of a nucleic acid encoding  
15 an individual polypeptide for which multiple mutations have been identified, a nucleic acid encoding Env gp120 can contain the deletion of up to eight amino acids of amino acid residues 144 through 151. A nucleic acid encoding Env gp120 can also contain the deletion of one  
20 or two amino acids of amino acid residues 192 and 193. A nucleic acid encoding Env gp120 can additionally contain the combination of deletion of up to eight amino acids of amino acid residues 144 through 151 and deletion of one or two amino acids of amino acid residues 192 and 193.  
25 Additionally, any combination of one or both of the deletion mutations can be combined with Arg at amino acid residue 143. The combination of one or both of the deletion mutations can also be combined with Ile at amino acid residue 153. Also, the combination of one or both  
30 of the deletion mutations can be combined with Val at amino acid residue 187. Additionally, the combination of one or both of the deletion mutations can be combined with one or two of the single amino acid substitutions.

In another embodiment in which nucleic acids encode a combination of amino acid mutations of polypeptides, the invention also provides a substantially pure nucleic acid or fragment thereof that encodes more than one polypeptide containing at least one mutation. A nucleic acid of the invention can therefore encode any one or more of the polypeptides selected from the group consisting of Nef, Env gp41, Pol protease, Env gp120 and Vpr polypeptides so long as the encoded polypeptides contain at least one of the mutations described above. For example, a substantially pure nucleic acid can encode a truncated Nef polypeptide containing amino acid residues 1 through 56 and Env gp41 containing Arg at amino acid residue 660. In another embodiment, a substantially pure nucleic acid can encode three or more of the polypeptides selected from the group consisting of Nef, Env gp41, Pol protease, Env gp120 and Vpr polypeptides, up to and including all of these polypeptides so long as at least one of the amino acid mutations described above is encoded by the nucleic acid. For example, a substantially pure nucleic acid can encode all of the polypeptides Nef, Env gp41, Pol protease, Env gp 120 and Vpr polypeptides having all of the amino acid mutations described above.

Further aspects of this invention are the substantially pure polypeptides or fragments thereof that are encoded by any of the above nucleic acid molecules. In one aspect of the invention, the substantially pure polypeptides or fragments thereof can be used to raise antibodies which are specific to the mutated proteins.

The invention provides a substantially pure HIV-1 polypeptide or a fragment thereof selected from the group of polypeptides consisting of Nef, Env gp41, Pol

protease, Env gp120 and Vpr, wherein the HIV-1 polypeptide contains at least one of the mutations found in HIV-1 provirus from L-2 cells. Such mutations found in HIV-1 provirus from L-2 cells are described above.

5 In one embodiment, the invention provides Nef polypeptides having one or more mutations in the Nef polypeptide of a HIV-1 provirus from L-2 cells. For example, the invention provides a substantially pure Nef polypeptide or fragment thereof comprising substantially  
10 the same sequence of amino acid residues 1 through 56 of a HIV-1 Nef polypeptide. The invention also provides a substantially pure Nef polypeptide or fragment thereof comprising substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Ala at amino acid  
15 residue 15. The invention additionally provides a substantially pure Nef polypeptide or fragment thereof comprising substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Lys at amino acid residue 19.

20 The invention further provides a substantially pure Nef polypeptide or fragment thereof comprising substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Val at amino acid residue 33. The invention also provides a substantially pure Nef  
25 polypeptide or fragment thereof comprising substantially the same amino acid sequence of an HIV-1 Nef polypeptide containing Asp at amino acid residue 54.

In addition to the Nef polypeptides having the individual mutations described above, the invention also  
30 provides a substantially pure Nef polypeptide or fragment thereof having any combination of one or more of the Nef polypeptide mutations. For example, the invention

provides Nef polypeptides having one or more of the amino acid substitution mutations described above in the full length Nef polypeptide. Furthermore the invention provides a truncated Nef polypeptide and a combination of one or more of the amino acid substitution mutations described above. For example, the invention provides a truncated Nef polypeptide containing amino acids 1 through 56 and Ala at amino acid residue 15. Additionally, the invention provides a truncated Nef polypeptide containing amino acids 1 through 56 and Lys at amino acid residue 19. Further provided is a truncated Nef polypeptide containing amino acids 1 through 56 and Val at amino acid residue 33. Also provided is a truncated Nef polypeptide containing amino acids 1 through 56 and Asp at amino acid residue 54.

In addition to the Nef polypeptide truncation mutants combined with a single amino acid substitution mutation, the invention also provides a truncated Nef polypeptide containing amino acids 1 through 56 and two or more of the amino acid substitution mutations described above. For example, the Nef polypeptide can contain amino acid residues 1 through 56 and Asp at amino acid residue as well as Val at amino acid residue 33. Furthermore, the invention provides a truncated Nef polypeptide containing amino acid residues 1 through 56 as well as Ala at amino acid residue 15, Lys at amino acid residue 19, Val at amino acid residue 33, and Asp at amino acid residue 54. Therefore, one aspect of the instant invention is the truncated HIV-1 Nef protein containing the amino acid substitutions set forth in SEQ ID NO: 46 (Fig. 4). The amino acid sequence of such a Nef polypeptide combining all of the Nef polypeptide mutations found in HIV-1 provirus from L-2 cells is shown in Figure 4 (SEQ ID NO:46).

The invention also provides a substantially pure Env gp41 polypeptide or fragment thereof comprising substantially the same amino acid sequence of an HIV-1 Env gp41 polypeptide containing Arg at amino acid residue 5 660. The invention additionally provides a substantially pure Pol protease polypeptide or fragment thereof comprising substantially the same sequence of amino acid residues 1 through 13 of an HIV-1 Pol protease polypeptide fused at the carboxyl-terminus to 10 substantially the same amino acid sequence as AsnAspArgGlyAlaThrLysGlySerSerIleArgTyrArgSerArg (SEQ ID NO:6).

The invention also provides a substantially pure Env gp120 polypeptide or fragment thereof comprising 15 substantially the same amino acid sequence of an HIV-1 Env gp120 polypeptide containing Arg at amino acid residue 143, Ile at amino acid residue 153, and Val at the amino acid residue corresponding to amino acid residue 187 of a wild type HIV-1 Env gp120 polypeptide 20 and absent amino acid residues corresponding to amino acid residues 144 through 151 and 192 through 193 of a wild type HIV-1 Env gp120 polypeptide.

The invention also provides a substantially pure Env gp120 polypeptide comprising substantially the 25 same amino acid sequence of an Env gp120 polypeptide with deletion of at least one and up to eight amino acids of amino acid residues 144 through 151. The invention additionally provides a substantially pure Env gp120 polypeptide comprising substantially the same amino acid 30 sequence of an Env gp120 polypeptide with deletion of one or both of amino acid residues 192 and 193.

Additional Env gp120 polypeptides are also provided in the present invention. For example, the invention provides an Env gp120 polypeptide comprising deletion of at least one and up to eight amino acids of amino acid residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193. Additionally, the invention provides Env gp120 polypeptides comprising deletion of at least one and up to eight amino acids of amino acid residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193 as well as Arg at amino acid residue 143. The invention also provides Env gp120 polypeptides comprising deletion of at least one and up to eight amino acids of amino acid residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193 as well as Ile at amino acid residue 153. Also, the invention provides Env gp120 polypeptides comprising deletion of at least one and up to eight amino acids of amino acid residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193 as well as Val at amino acid residue 187.

The invention further provides Env gp120 polypeptides comprising deletion of at least one and up to eight amino acids of amino acid residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193 as well as two of the amino acid substitution mutations. For example, the invention provides Env gp120 polypeptides comprising deletion of at least one and up to eight amino acids of amino acid residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193 as well as Arg at amino acid residue 143 and Ile at amino acid residue 153. The invention also provides Env gp120 polypeptides comprising deletion of at least one and up to eight amino acids of amino acid

residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193 as well as Arg at amino acid residue 143 and Val at amino acid residue 187.

The invention further provides Env gp120 polypeptides  
5 comprising deletion of at least one and up to eight amino acids of amino acid residues 144 through 151 and deletion of one or both of amino acid residues 192 and 193 as well as Ile at amino acid residue 153 and Val at amino acid residue 187.

10 The invention also provides a substantially pure Vpr polypeptide or fragment thereof comprising substantially the same sequence of amino acid residues 1 through 17 of an HIV-1 Vpr polypeptide.

As discussed above for Nef polypeptide and Env  
15 gp120, any of the mutations in an individual polypeptide can be used alone or in combination with one or more additional mutations in the same polypeptide. Thus, the invention provides a substantially pure HIV-1 polypeptide or fragment thereof selected from the group consisting of  
20 Nef, Env gp41, Pol protease, Env gp120 and Vpr that contain two or more of the mutations described above in a single individual polypeptide.

The invention thus provides a substantially pure HIV-1 polypeptide or fragment thereof selected from  
25 the group of polypeptides consisting of Nef, Env gp41, Pol protease, Env gp120 and Vpr, wherein the HIV-1 polypeptide or fragment thereof comprises at least one of the mutations described above and can, where more than one mutation has been described in an individual  
30 polypeptide, comprise any combination of two or more mutations, including as many as all of the mutations described for an individual polypeptide.

Any combination of one or more of the HIV-1 polypeptides containing any combination of one or more of the mutations described above can be expressed together as desired. The choice of combination of polypeptides and the combination of mutations in the polypeptides depends on the needs of the investigator.

The invention also provides nucleic acids encoding substantially the same amino acid sequence of the mutant HIV-1 polypeptides described above. Any nucleic acid that encodes any combination of single mutations in a polypeptide, multiple mutations in a polypeptide or a combination of two or more polypeptides of the invention are also provided.

The genes of HIV-1 provirus from L-2 cells described above were isolated using PCR amplification as described in Example I. However, the nucleic acid molecules described above can be isolated from L-2 cells by any of a variety of methods well known in the art. For example, in addition to PCR amplification of nucleic acids, other methods well known in the art regarding cloning of DNA from an organism can be used to isolate HIV-1 provirus DNA (Sambrook et al., Eds., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Springs Harbor Laboratory Press (1989), which is herein incorporated by reference). Furthermore, the nucleic acids of the invention, which are set forth above, can be synthesized by chemical means (See, Blackburn and Gait, Eds., Nucleic Acids in Chemistry and Biology, 2nd Ed., Oxford University Press (1996), which is herein incorporated by reference).

The proteins and peptides encoded by said nucleic acids can be isolated and purified from L-2 viral

particles (see, for example, Deutscher, Ed., Methods in Enzymology: Guide to Protein Purification, Vol. 182, Academic Press, Inc., San Diego, CA (1990), herein incorporated by reference), or chemically synthesized or  
5 expressed in a recombinant organism by methods well known in the art, such as Stewart and Young, Eds., Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Company (1984); Kriegler, M., Ed., Gene Transfer and Expression: A Laboratory Manual, Stockton Press, New York, NY (1990);  
10 and Jones, J., Ed., The Chemical Synthesis of Peptides, Oxford University Press, Oxford (1994), all of which are herein incorporated by reference. For example, the substantially pure nucleic acids of the invention can be expressed recombinantly in bacterial or eukaryotic cells  
15 and subsequently purified.

As non-infectious particles, the L-2 viral particles can be used as immunogens to induce a host immune response against an HIV infection. More specifically, the immunogens can be used for the  
20 prevention or reduction of apoptotic cell lysis caused by HIV infection.

The L-2 viral particles can be produced by methods well known in the art. For example, non-infectious viral particles can be produced by growing L-2  
25 cells in culture under conditions such that protease-defective L-2 HIV particles are produced. Such particles are then separated from L-2 cells by, for example, centrifugation of the L-2 cell supernatant and recovering the particles in the pellet. Such methods are described  
30 further below in the Examples as well as in, for example, Ohki et al., J. Acquired Immun. Def. Syn. 4:1233-1240 (1991).

The non-infectious L-2 viral particles can be used directly as an immunogen or, alternatively, can be further treated to ensure multiple, additional levels of viral particle inactivation. Additional inactivation treatment is a precautionary measure to provide further confidence and safety levels against possible commination of non-L-2 HIV viral particles. Methods of viral particle inactivation are well known in the art and include, for example, treatment with gamma-radiation, beta-propiolactone and gluaraldehye. These methods are described further below in the Examples. Moreover, the production and use of immunogens is taught, for example, in Salk et al., U.S. Patent No. 5,256,767, issued October 26, 1993. Therefore, the invention provides an immunogen consisting of non-infectious, protease-defective particles produced by L-2 cells. The invention also provides inactivated, protease-defective particles produced by L-2 cells. The non-infectious, protease-defective particles can also be produced by non-L-2 cells which harbor the L-2 provirus genome.

As described above, the L-2 viral particles contain a number of mutations which render the particles non-infectious. These mutations can be advantageously exploited to engineer a variety of non-infectious viral particles as specific immunogens against preselected types of HIV viruses. For example, it is not necessary to have all of the mutations described herein for the particle to be non-infectious and therefore useful as an immunogen. Instead, immunologically effective immunogens can be prepared from modified forms of the L-2 viral particles containing less than all of the mutations described herein so long as the modified L-2 viral particle contains a mutation that renders the particle non-infectious. Such mutations can be, for example, any

one of the mutations identified in the *Pol* protease, *Nef*, or *Env* gp41, for example. These mutations can be combined with the L-2 *Env* gp120 polypeptide or the L-2 *Env* gp120 mutants described herein to produce non-infectious viral particles for vaccinating against HIV. Other mutant polypeptides such as the L-2 *Vpr* or the L-2 *Vpr* mutants described herein can also be included within the viral particles to augment the non-infectious phenotype of the modified L-2 viral particles. Those skilled in the art will know or can determine which mutation or combination of mutations are sufficient to render the particle non-infectious given the mutant sequences and teachings described herein.

Alternatively, the L-2 mutations identified in the *Pol* protease, *Nef*, or *Env* gp41 polypeptides can be combined with *Env* gp120 polypeptides from other HIV virus sub-types, or clades, to produce non-infectious viral particles. These particles can similarly be used as vaccines against the various HIV clades. For example, there are two primary groups of HIV clades, termed M and O, which characterize the infective phenotype of the virus. For group M, there are eight clades (A through J). The L-2 viral particles are characterized as being within the B clade of Group M, Betts et al., J. Virology 71:8909-8911 (1997).

The L-2 viral particle are characterized as being within the O clade having subgroup E. Substituting *Env* gp120 derived from different clades or clade subgroups into non-infectious L-2 viral particles will render a modified L-2 particle specific for the substituted *Env* gp120 clade. These clade-specific, modified L-2 particles can then be used as immunogens for the prevention and treatment of an HIV infection.

As with the modified L-2 viral particles described above, immunogenically effective immunogens can be prepared from the clade-specific forms of the modified L-2 viral particles that contain less than all of the L-2 mutations described herein so long as the particles contain at least one of the mutations that renders the particle non-infectious. Such mutations can be, for example, any one of the mutations identified in the *Pol* protease, *Nef*, or *Env* gp41, for example. Additionally, the mutations described in the *Vpr* protein can be optionally incorporated. Those skilled in the art will know or can determine which mutation or combination of mutations are sufficient to render the clade-specific viral particles non-infections given the mutant sequences and teachings described herein.

Production of the modified L-2 particles or the modified, clade-specific particles described above can be performed using methods well known in the art given the L-2 nucleotide and amino acid sequences and the teachings described herein. Such methods can include, for example, the construction and expression of recombinant modified L-2 genomes so as to contain a desired combination of the L-2 mutations which confer the non-infectious phenotype. The construction of such desired combinations can be performed by, for example, site directed mutagenesis or by polymerase chain reaction (PCR) using mutagenic primers. Other well known methods exist in the art and can similarly be used to generate the desired combination of L-2 mutations within to produce a modified L-2 genome or a modified, clade specific genome. Once produced, the genomes can be stably introduced into compatible cells, such as T cells, to harbor the provirus and effect the production of the modified viral particles. Such methods are well known in the art and can be found described in,

for example, Sambrook et al., Eds., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press (1989), and Ausubel et al., Eds., Current Protocols in Molecular Biology, John Wiley & Sons, (1987). The

5 non-infectious, modified L-2 particles, or the non-infectious, modified particles that exhibit clade-specific phenotypes can be isolated and used directly as immunogens or be subjected to further inactivation procedures as described above prior to use as immunogens

10 for the prevention or treatment of apoptotic cell lysis mediated by HIV.

Specific examples of non-infectious, modified L-2 viral particles include particles containing at least an expressible *Nef* or *Pol* protease mutant as described

15 previously. For example, the *Nef* mutant can contain the stop codon at position 57 and/or contain one or more of the nucleotide and corresponding amino acid mutations set forth in Figures 3 and 4, (SEQ ID NOS:45 and 46, respectively). The *Pol* protease mutant can contain one

20 or more of the nucleotide and corresponding amino acid mutations set forth in Figure 1 (SEQ ID NO:5 and 6, respectively). Any of the *Nef* or *Pol* protease mutants can additionally be combined with the various *Vpr*, *Env* gp41 or *Env* gp120 mutants previously describe to generate

25 a non-infectious, modified L-2 viral particle of the invention. Similarly, such mutations can be constructed with non-L-2 *Env* gp120 polypeptides to produce non-infectious, modified L-2 particles that are clade specific.

30 Any of the previously described non-infectious L-2 viral particle immunogens, including modified forms and clade-specific forms, can additionally be combined with other HIV immunogens to further augment an immune

response directed to HIV. Additional HIV immunogens can include, for example, inactivated forms of the virus, virus which is devoid of its outer envelope proteins, as well as soluble HIV polypeptides or fragments thereof.

5 These additional HIV immunogens that can be combined with the non-infectious L-2 particles and modified forms thereof, can be derived from HIV-1, HIV-2 or both, for example. Such combined immunogens can similarly be formulated in an adjuvant for administration. Immunogens  
10 for the induction of an immune response against HIV are well known in the art. A specific example of an immunogen which is devoid of the *Env* gp120 polypeptide is described, for example, in Salk et al., supra.

Therefore, the invention provides a method for  
15 the reduction or prevention of apoptotic cell lysis mediated by HIV. The method consists of administration of a non-infectious L-2 particle or non-infectious modified L-2 particle to a human who is either seropositive or seronegative for HIV, and particularly in  
20 those patients where the apoptotic lysis is induced by protease-defective particles. Additionally, the invention provides for methods of stimulating the immune system of a human. Such a method would comprise administering one of the above non-infectious L-2  
25 particle or modified L-2 particle immunogens, or a composition including such immunogens in an immunologically effective amount to a human. Again, the human could be seronegative or seropositive for HIV.

The non-infectious immunogens of the invention  
30 are administered to a host exhibiting or at risk of exhibiting an autoimmune response. Definite clinical diagnosis of AIDS or AIDS related complex (ARC) warrants the administration of the relevant immunogen.

Prophylactic applications are warranted where an HIV infection precedes the onset of overt clinical disease or where the individual has been at risk of exposure to the virus. Thus, individuals with any history of infection  
5 or predicted to be at risk by reliable prognostic indicators can be treated prophylactically to interdict immunodeficiency mechanisms prior to their onset.

The non-infectious immunogens of the invention can be administered in many possible formulations,  
10 including pharmaceutically acceptable mediums. The immunogens can include or be administered in conjunction with an adjuvant, of which several are known to those skilled in the art. After initial immunization with the non-infectious immunogen, further boosters can  
15 additionally be provided. The non-infectious immunogens are administered by conventional methods, in dosages which are sufficient to elicit an immunological response. Such dosages are known and also can be easily determined by those skilled in the art.

20 Briefly, individuals who are candidates for immunization can be effectively treated by active immunotherapy using a non-infectious immunogen prepared from the L2-viral particles or modified forms thereof. The dose is selected so as to be immunologically  
25 effective, and is generally between about 1 to about 100  $\mu\text{g}$  of protein, more preferably about 30  $\mu\text{g}$  of protein.

Active immunization is implemented and preferably repeated once at a minimum interval of at least 90 days, although additional boosts may be  
30 appropriate according to changes in the immunocompetence level, based, for example, on a decline in antibodies to HIV gene products other than outer envelope proteins.

Such immunization is preferably accomplished initially by intramuscular injection followed by intradermal injection, although any combination of intradermal and intramuscular injections may be used.

5                    Preferably, the immunoresponsiveness or immunocompetence of the seropositive individual is determined prior to immunization in order to determine an appropriate course of therapy. As a method of such determination, individuals' sera can be screened for the  
10 presence of antibodies to p24 (as by means of ELISA), for TRI antibody and/or for the level of T<sub>4</sub> cells by methods well known in the art. Individuals exhibiting indicators of low immunocompetence, such as low p24 or RTI antibody titers or low numbers of T<sub>4</sub> cells, are appropriate  
15 candidates for active immunotherapy. Additionally, active immunotherapy can be combined with other therapies known in the art including, for example, passive immunotherapy.

                  Seronegative individuals can be vaccinated in  
20 order to induce immunoprotective factors to prevent infection. Preferably, the vaccine is administered initially by intramuscular injection followed by a booster injection given either intramuscularly or intradermally. A physiologically effective dose,  
25 preferably in the range of 1 to 100 µg and more preferably about 30 µg of immunogen is provided per dose. The vaccine can be administered in conjunction with an adjuvant, such as a water-in-oil type adjuvant. Various appropriate adjuvants are well known in the art as  
30 reviewed by Warren and Chedid, CRC Critical Reviews in Immunology 8:83 (1988).

These methods as well as other modes of administration are well known in the art and can be used for the prevention or treatment of an HIV infection using the non-infectious immunogens described herein.

5           The nucleic acid molecules discussed above can be used as nucleic acid probes or standards in either diagnostic or in PCR procedures. Such diagnostic methods can be used to detect the previously described mutant DNA sequences in cells and extracellular fluids. Methods of  
10 detection by hybridization with nucleic acid probes are well known in the art and are described in, for example, Hames and Higgins, Eds., Nucleic Acid Hybridisation: A Practical Approach, Oxford University Press, Oxford (1991), and Innis et al., Eds., PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc. (1990).  
15 Various formats known in the art can be used for detection of L-2 specific mutations by nucleic acid hybridization. The choice of such formats will vary depending on the particular application and need of the  
20 diagnostic procedure. Formats include, for example, solid-phase hybridization and solution hybridization. Specific examples of solid-phase hybridization formats for diagnostic procedures include the use of diagnostic chips containing two-dimensional arrays of  
25 oligonucleotides, multi-well formats such as an ELISA and blot hybridization procedures. Such formats and various permutations thereof are well known in the art.

          Similarly, the polypeptides above could be used as assay standards in immunoassays kits, and could be  
30 employed to raise antibodies for immunoassays to detect the above mutated polypeptides and organisms containing them. Methods for raising monoclonal and polyclonal antibodies specific to such polypeptides are well known

in the art. (See, for example, Harlow and Lane, Eds., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory (1988). Moreover, the production of monoclonal antibodies is described in detail in U.S. Patent Nos. RE 32011 and 4,411,993. Antibodies to L-2 mutant polypeptides can additionally be generated using combinatorial methods well known in the art. Such methods include the generation and screening of recombinant antibody library repertoires for antibodies specific to an antigen of interest, including any of the L-2 mutant polypeptides described herein (see, for example, Huse et al., Science 246:1275-1281 (1989)). Monoclonal antibodies specific for the L-2 polypeptide described herein can additionally be modified by recombinant methods known in the art to incorporate human antibody framework sequences. Such methods are well known in the art and are described in, for example, U.S. Patent No. 5,585,089 to Queen et al.

Once suitable antibodies or binding proteins have been obtained, they may be isolated or purified by many techniques well known to those of ordinary skill in the art (see Antibodies: A Laboratory Manual, Harlow and Lane (eds.), supra). Suitable techniques include peptide or protein affinity columns, HPLC or RP-HPLC, purification on protein A or protein G columns, or any combination of these techniques.

Therefore, the invention provides monoclonal and polyclonal antibodies, and fragments thereof specific for L-2 mutant polypeptides, as well as chimeric and humanized antibodies and fragments specific for such polypeptides. Such antibodies can be additionally modified with, for example, a label or a therapeutic agent for the diagnosis or treatment of HIV.

The antibodies raised against the above-described L-2 proteins can be used in quantitative, semi-qualitative, or qualitative diagnostic assays. Such assays, for example, can be in RIA, IRMA, and ELISA formats. Examples of such formats include a competitive or sandwich arrangement. See, for example, David et al., U.S. Patent No. 4,486,530, issued December 4, 1984; Motagnier et al., U.S. Patent No. 4,708,818, issued November 24, 1987; Montagnier et al., U.S. Patent No. 5,135,864, issued August 4, 1993; Gallo et al., U.S. Patent No. 4,647,773, issued March 3, 1987; Gallo et al., U.S. Patent No. 4,520,113, issued May 28, 1985; and Gallo et al., U.S. Patent No. 4,652,599, issued March 24, 1987, all of which are herein incorporated by reference. In a competitive format, the instant antibodies are incubated with the sample to be analyzed and a measured quantity of labeled, competing analyte. The antibody in competitive assay is usually bound to a solid support, or is capable of being bound to such a support, as discussed below. Such binding to a solid support can take place before or after the instant antibody is incubated with the sample suspected of containing one of the instant proteins. In a sandwich format, two antibodies, each specific for the analyte, are reacted one at a time or simultaneously with the sample to be analyzed. Typically, one of the instant antibodies will be either be labeled with a detection means or capable of being so and the other will either be attached to a solid support or capable of being so. After the antibody-analyte-antibody sandwich complex is formed, the detection means is measured. Alternatively, after the complex is formed, the complex is then attached to a solid support or to a detection means, or both.

Binding to the solid support can be accomplished by an antibody that in turn binds to one of

the antibodies of the complex, the natural or covalent attachment of the unlabeled antibody to the support, or by the reaction of another ligand-receptor pair, such as biotin-avidin. In the latter case, biotin is bound  
5 either to the constant region of the antibody or to the solid support and avidin is bound to the remaining component. In either the competitive or sandwich format, there are usually wash, aspiration and possibly filter procedures after the incubation of the sample and  
10 following the additions of each reagent.

The antibodies used in the above diagnostic assays can use, for example, one or more of the instant polyclonal or monoclonal antibodies, mixtures thereof, and the corresponding functional fragments. For example,  
15 in a sandwich assay, the two antibodies used to form the complex can either both be polyclonal or monoclonal or functional fragments thereof. Alternatively, one monoclonal and one polyclonal can form the sandwich. Similarly, in the competitive format, the antibody can be  
20 one or more monoclonal or polyclonal antibodies, or functional fragments thereof.

The detection means are well known in the art. Such detection means can be, for example, enzymes, radioisotopes, fluorogens, chromogens, metallic or  
25 non-metallic colloids, colored liposomes, colored or colorable particles, and the like. Thus, the detection means may be immediately visible after the analyte complexes with the instant antibodies in an assay, or further steps may need to be taken to utilize the  
30 detection means. Such steps include UV and visible spectrophotometry, fluorimetry and analysis by radiation counters. Examples of such detection means include alkaline phosphatase/para-nitrophenyl phosphate; horse

radish peroxidase/aminoethylcarbazole, colloidal gold, colloidal silver, colloidal selenium, hydrophobic dyes, colored latex microparticles, carbon sols, rhodamine, fluorescein, luminol, luciferin, umbeliferone, <sup>125</sup>Iodine, <sup>131</sup>Iodine, tritium, <sup>32</sup>Phosphorous, and the like. These  
5 detection means can be coupled to the either the analyte (competitive assays) or to an antibody (sandwich assays) by means well known in the art. Suitable detection means and methods of making them are further discussed, for  
10 example, in Tom et al., U.S. Patent No. 4,366,241, issued December 28, 1982; and in Campbell, U.S. Patent No. 4,703,017, issued October 27, 1987.

The optional solid supports for the assays can be bibulous or nonbibulous. Examples of bibulous  
15 supports include nitrocellulose, nylon, filter paper, and the like. Non-bibulous supports are ones that do not necessarily effect a chromatographic separation of the components as they advance along the solid support. Examples of such non-bibulous supports include plastic  
20 materials such as high density polyethylene, polystyrene, polyvinyl chloride, mixtures of such high density plastics, or bibulous material rendered non-bibulous by the application of non-blocking materials, such as detergents and proteins. These and other supports and  
25 methods of use are known in the art.

The invention also provides diagnostic kits comprising the L-2 specific antibodies. In addition, such kits can contain amounts of reagents for use in assaying for an analyte. Such reagents can be substrates  
30 when enzymes are used as the detection means, as well as ancillary reagents like stabilizers, extraction reagents, buffers, wash solutions, and the like. The relative amounts of the various reagents vary widely, to provide

for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. The reagents can be provided as dry powders, usually lyophilized, including excipients, which on dissolution  
5 will provide for a reagent solution having the appropriate concentrations for performing the assay. The kit can also be contained in packaging material, such as air-tight foil, or various external containers known in the art. Such external containers can contain the  
10 device, reagents, and the instructions for use of the assays.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also included within  
15 the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

#### EXAMPLE I

This Example describes the determination of the  
20 genetic makeup of the HIV-1 provirus in L-2 cells.

In initial studies the cleavage pattern of the HIV-1 Gag polypeptide expressed in *Escherichia coli* transformed with recombinant L-2 gag containing an HIV-1 wild-type *pol* protease gene was found to be similar to  
25 that of parental HIV-1 (LAI) particles. Therefore, the L-2 cell provirus was sequenced at other regions of the genome. Other regions of the L-2 cell proviruses genome that were sequenced included *pol* protease; *env* gp120 (SU) and gp41 (TM); and the accessory region genes such as  
30 *vif*, *vpu*, *vpr*, and *nef*. The control was the provirus in MOLT-4 cells (ATCC) persistently infected with HIV-1

(LAI), which was designated as MO/LAI was used as a control.

The polymerase chain reaction (PCR) amplification of total cellular DNA extracted from L-2 and MO/LAI was performed as previously described (Kameoka et al., Virus Genes, 12:117-129 (1996), utilizing 30 cycles of 1 min at 94°C, 4 min at 60°C, followed by a final polymerization step for 10 min at 72°C, with a GeneAmp XL PCR kit (Perkin-Elmer Corporation, Foster City, CA). The primers used at each region are summarized in Figure 2 (Adachi et al., J. Virol, 59:284-291 (1986). The PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The expected bands were ligated into a pBluescript II SK(-) vector (Stratagene Cloning Systems, La Jolla, CA). Nucleotide sequencing of the amplified DNA segments was carried out using ( $\gamma$ -<sup>32</sup>P)ATP-labeled T3, T7, or synthetic primers by cycle sequencing according to the protocol described for the CircumVent thermal cycle sequencing kit (New England BioLabs, Beverly, MA).

The nucleotide sequences of provirus genes in the L-2 cells were compared with those determined from MO/LAI as well as the reported HIV-1 (LAI) sequences (GenBank accession number KO2013) (Wain-Hobson et al., Cell, 40:9-17 (1985), (see Figure 1). Comparison of the nucleotide sequences of provirus genes to the nucleotide sequences HIV-1, LAI and HIV-1 MO/LAI genes reveals significant mutations in the *pol* protease, *env* gp41, and *nef* genes for L-2 proviral DNA. Specifically, the protease gene had an insertion mutation of T at nucleotide position 42, resulting in the appearance of a stop codon at amino acid residue 30. This explains the protease-defective nature of L-2 particles, as evidenced

by an inability to cleave the Pr55<sup>gag</sup> precursor inside L-2 cells. For the TM envelope glycoprotein, gp41, a 1-base substitution was found at nucleotide 7781 (A to G; corresponding to nucleotide 1979 in Figure 1), resulting  
5 in an amino acid substitution at amino acid residue 660, from a lysine to an arginine. The *nef* gene also had a nonsense mutation at nucleotide 8559 (G to A; corresponding to nucleotide 170 in Figure 1), resulting  
10 in the appearance of a stop codon at amino acid residue 57, as well as other mutations set forth in Figure 3. All three of these changes in the protease, gp41, and *nef* genes were specific for L-2 proviral DNA.

In contrast, the *vpr* gene was similarly truncated for both L-2 and MO/LAI proviral DNAs owing to  
15 a 1-base substitution at nucleotide 5194 (G to A; corresponding to nucleotide 54 in Figure 1), resulting in the appearance of a stop codon at amino acid residue 18. The *vif* and *vpu* genes were identical for both L-2 and MO/LAI proviral DNAs. Also, the sequencing of *env* gp120  
20 revealed no apparent differences between L-2 and MO/LAI although both showed V1 and V2 domains that contained deletions of either eight or two amino acids, respectively, as compared to the reported HIV-1 (LAI) sequence. Therefore it is possible that these deletions  
25 in gp120 could have occurred during the long-term maintenance of HIV-1 (LAI) in different T cell lines during the instant studies, with no apparent effect on their infectivity.

The data presented here point to the specific  
30 involvement of one or more mutation(s) in the L-2 provirus *pol* protease, *env* gp41, and/or *nef* genes as the cause for the production of defective, but highly syncytium- and apoptosis-inducing particles. In

particular, the amino acid mutation at Env gp41 accounts for the higher functional activity of protease-defective particles. Furthermore, the gp120 polypeptide deletions of 8 and 2 amino acids corresponding to SEQ ID NOS:18 and 24 (Figure 1), respectively, are critical in defining the pathogenicity of L-2 particles in the context of the L-2 Nef, protease, gp41, or Vpr mutant-derived polypeptides. These L-2 particles are responsible for causing the highly pathogenic end-stages of HIV-1 disease leading to ARC and AIDS. Thus, it is important to stimulate the immune system to attack any defective L-2 particles that accumulate during infection. The use of inactivated L-2 particles, optionally in conjunction with other HIV-1 or HIV-2 immunogens, or both, nucleic acid sequences and fragments, polypeptides and fragments thereof, and antibodies, as described in this invention, alone or in combination thereof, will remove potentially harmful defective L-2 particle from HIV-1 infected tissues of individuals, leading to an improved outcome.

These results show that HIV-1 provirus from L-2 cells have mutations in the pol protease, nef, env and vpr genes that encode the pol protease, Nef, gp41, gp120 and Vpr polypeptides and result in mutations in these polypeptides.

## EXAMPLE II

### Production of Non-infectious, L-2 Particles or Modified Forms Thereof

This Example describes the production of non-infectious L-2 particles or modified forms thereof. The method of production of non-infectious, L-2 particles or modified forms thereof has essentially been described previously with some modifications (Prior et al., (1995);

Prior et al. (1996)). The modifications are related to the cell culture and downstream recovery conditions to improve both production levels and purification of virus from the growth medium containing 5-10% fetal bovine serum.

The production method for L-2 particles will mimic those used for HIV-1 described below. The initial production steps involve anion exchange chromatography. Briefly, the cell line used is the L-2 cell line. After multiple cell culture expansions with cell densities in the range of one to two million cells per milliliter, the cellular debris is separated from virus by dead-end filtration using a 1.2  $\mu$ M cellulose ester membrane. The filtered supernatant containing L-2 particles is inactivated by addition of  $\beta$ -propiolactone (1:2000 v/v), concentrated by tangential flow ultrafiltration using a 300,000 molecular weight (MW) cut-off polysulphone membrane and the concentrates are stored at  $-70^{\circ}\text{C}$ . This hold step permits analysis of in-process material in an infectivity assay to determine that infectious virus is nondetectable before further processing. Thawed concentrates are pooled, filtered and applied to TMAE Fractogel (Merck, Darmstadt, Germany), washed with 0.5M NaCl at pH 6.5 and eluted in 1.0M NaCl at pH 6.5. After immediate dilution to reduce NaCl concentration to about 0.15M, product is applied to Q-Sepharose (Pharmacia, Inc., Piscataway, NJ, USA), washed in 0.7M NaCl and eluted in 1.0M NaCl, all at pH 6.5. Following concentration/diafiltration (polysulphone, 3000 MW cut-off), product is centrifuged and the pellet is resuspended, then frozen and subjected to  $\gamma$  irradiation on dry ice. The combined inactivation steps,  $\beta$ -propiolactone and  $\gamma$  irradiation, can be validated to attain a  $> 17$  log viral inactivation capability.

Briefly, the pooled filtrate containing product is inactivated with  $\beta$ -propiolactone ( $\beta$ PL) and subjected to ultrafiltration. Briefly, following pH adjustment of the pooled filtrate to  $7.3 \pm 0.1$ ,  $\beta$ PL is added while stirring to a final concentration of 1:2,000 (v/v). After stirring for one hour, the preparation is transferred to a second mixing vessel to ensure complete viral inactivation and incubated at  $4^{\circ}\text{C}$  for 18-24 hours with continuous stirring. The temperature of the filtrate is raised to  $37^{\circ}\text{C}$  and maintained at that temperature for approximately five hours to hydrolyze residual  $\beta$ PL into an isomer of lactate and betapropionic acid derivatives. Approximately 10-200 liters of post  $\beta$ PL-treated filtrate is concentrated to 2.5-3.5 liters using a polysulfone 300,000 molecular weight (MW) cutoff membrane and diafiltered against 10-20 volumes of phosphate buffered saline. This step removes more than 95% of the growth medium-derived polypeptide from the permeate, including approximately 50% of the starting p24 antigen as measured by ELISA. In this MW fraction (less than 300 kDa), p24 antigen is not associated with intact particles. The MW cutoff properties of the membrane achieves almost total retention of intact particles. The concentrate is stored frozen at  $-70^{\circ}\text{C}$ . This is a critical hold step because individual concentrates, derived from a single expansion of cell culture, can be thawed and pooled to the required lot size for subsequent chromatography. This hold step can be used for quality control to determine which concentrates to release for further processing, after conducting sterility testing and after verifying that a cell culture is free of adventitious virus and mycoplasma. This in-process check before pooling can prevent compromising large lot sizes, thus avoiding economic risk while maintaining the safety and integrity of a process.

Validating the use of  $\beta$ PL for inactivating HIV-1 is another critical safety measure. To determine the effectiveness of inactivating HIV-1 with  $\beta$ PL, a high concentrate of infectious virus is used at 0 time (starting time, pre- $\beta$ PL).  $\beta$ PL is added (1:2,000 v/v), and the decay in infectivity and  $\beta$ PL is evaluated. After adding  $\beta$ PL, samples are taken hourly,  $\beta$ PL activity is immediately neutralized with sodium sulfite, and the material is maintained at  $-70^{\circ}\text{C}$  pending analysis of the infectivity and  $\beta$ PL assays. Evaluation of inactivation is computed by subtracting the final log concentration at the time point measured from the log concentration of infectious HIV-1 at 0 time (pre- $\beta$ PL). Previous unpublished work has shown that, within 12 hours, infectious virus is either nondetectable or at trace levels, and absolute inactivation is confirmed 18 hours after  $\beta$ PL is added. Because the half-life of  $\beta$ PL in production medium is approximately 19 hours, sufficient residual  $\beta$ PL exists to continue inactivation beyond the 12-18 hours during which infectious virus is nondetectable. Therefore, to evaluate the inactivation potential for the entire production time of  $\leq 53$  hours, a fresh concentration of infectious HIV-1 is added in the form of an infectious spike to the  $\beta$ PL-treated material after 12 hours to determine the cumulative inactivation. Separate validation runs can be performed in duplicate.

For large scale cell culture and harvest of L-2 particles, the cell line used is L-2 cells. Briefly, frozen vials of L-2 cells are thawed and cultures are initiated. The culture medium throughout most of the expansion process is 10% fetal bovine serum (FBS) in RPMI-1640 with 25 mM HEPES - N-(2-hydroxyethyl)piperazine-N'-2-ethanesulphonic acid. As the cells divide, the culture volume is expanded to

larger vessels and sparged daily with 5% CO<sub>2</sub>/air. In the final expansion (from approximately 72L to a final volume of 144 L), the FBS content is reduced to about 7.5% with 5.0% FBS in RPMI-1640 and 25mM HEPES. Virus is separated from intact cells and cellular debris using a 1.2-µm cellulose ester filter. The pooled filtrate-containing product is cooled and maintained at 4°C. The entire cell culture production cycle, from the initial thaw of cells to the harvest of the final expansion takes approximately 35-45 days.

For cobalt irradiation and final formulation, the frozen, postchromatography product is removed from a biosafety level 3 (BSL-3) containment facility and on dry ice is subjected to 4.5-5.5 Mrad of cobalt irradiation. The objective is to optimize virus inactivation potential and minimize alteration to polypeptide structure to produce an immunogen. Cobalt irradiation inactivates virus by shearing nucleic acid into pieces of approximately 200 base pairs (bp), as determined by polymerase chain reaction (PCR), rendering virus biologically dysfunctional. The HIV-1 inactivation efficiency of cobalt irradiation is evaluated by aliquoting a given preparation of starting high-tier infectious virus and subjecting each sample maintained at -70°C to a single dose level of irradiation. Individual aliquots are subjected to 1.0, 2.0, 3.0, 4.0, 5.0 Mrad and measured for levels of infectivity. The study is performed in triplicate using three separate lots of infectious virus. The log reduction of infectivity is determined in primary peripheral blood mononuclear cells using the starting infectious titer following 5 Mrads of irradiation. The mean reduction in infectivity is expected to be 7 logs.

Cobalt irradiation also functions to sterilize the product. This is important because, unlike polypeptide solutions, purified HIV-1 particles such as non-infectious, L-2 particles or modified forms thereof cannot be subjected to sterile filtration techniques. A 0.2  $\mu$ m filter retains HIV-1 particles. Following irradiation, product is thawed, diluted with saline to a required dose, and emulsified with an equal volume of incomplete Freund's adjuvant. Syringes are filled for intramuscular administration with final bulk product - an off-white, viscous emulsion. Consistent sterility of product is maintained by performing formulation steps in an aseptic area supplied with high quality air such as class 100 filtered air maintained under positive pressure.

Inactivated purified product is thawed, polypeptide content determined, diluted in saline and emulsified with an equal volume of incomplete Freund's adjuvant (IFA) (Seppic Inc. Paris, France), to achieve a water:oil ratio of 1:1. All liquids and containers are maintained at 2-8°C to attain an optimal viscosity. The bulk product is filled into syringes for intramuscular administration.

To determine the purity of the final viral particle product, analytical gel-filtration chromatography is used. Analytical gel-filtration chromatography CL6B resin (Pharmacia Inc.) is packed into a 16 mm x 310 mm low-pressure Pharmacia XK16 column. The total column volume is about 60 ml. Low-pressure chromatography conditions are employed during the separation procedure to ensure that the virion remains intact, including use of a non-denaturing buffer system. The running buffer is 0.1M Tris and 0.5M NaCl, pH 8.0.

This ionic strength eliminates any adsorption of the virion onto the resin. The CL6B resin is highly crosslinked and has a MW fractionation range between 10 and 4000 kDa (molecules with a MW>4000 kDa are eluted in the void volume). The column is controlled by a Perspective Biosystems BioCAD 60 control system (Cambridge, MA. USA) at a flow rate of 2 ml min. Chromatographic profiles are monitored at two wavelengths: 230 and 280 nm. To determine HIV-1 antigen recovery and calculate mass balance, approximately 1.5 ml fractions are collected.

The non-infectious, L-2 particles or modified forms thereof produced by the procedure described above can be used for the prevention or treatment of diseases caused by HIV infection.

Throughout this application various publications have been referenced within parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

Although the invention has been described with reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention. It should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A substantially pure nucleic acid molecule or fragment thereof that encodes a truncated Nef protein comprising a nucleic acid sequence that encodes  
5 substantially the same sequence of amino acid residues 1 through 56 of an HIV-1 Nef protein.

2. The nucleic acid molecule of claim 1 wherein said nucleic acid sequence is selected from the group consisting of:

10 a nucleic acid molecule that encodes substantially the same amino acid sequence set forth in SEQ ID NO:46; and

15 a nucleic acid molecule that encodes substantially the same sequence of amino acid residues 1 through 56 of an HIV-1 Nef peptide wherein amino acid residue 54 is aspartic acid.

3. The nucleic acid molecule of claim 2 wherein said nucleic acid sequence is selected from the group consisting of:

20 (a) nucleotides 1 through 168 of SEQ ID NO:45; and

(b) nucleotides 1 through 168 of an HIV-1 Nef peptide wherein nucleotide 161 is adenine.

4. A substantially pure fragment of the nucleic acid molecule of claim 3 comprising a nucleic acid sequence that has one or more of the following amino acid residues of SEQ ID NO:45: guanine at nucleotide 43,  
5 adenine at nucleotide 56, thymine at nucleotide 98, adenine at nucleotide 111, and adenine at nucleotide 161 and can be used to specifically detect the nucleic acid molecule of claim 3(a).

5. A substantially pure mutated HIV-1 Nef  
10 protein fragment, comprising a peptide encoded by the nucleic acid molecule fragment of claim 4.

6. An antibody or fragment thereof that specifically binds to the protein fragment of claim 5.

7. A substantially pure truncated Nef protein  
15 or fragment thereof comprising substantially the same sequence of amino acid residues 1 through 56 of an HIV-1 Nef protein.

8. The truncated Nef protein of claim 7  
wherein said sequence is selected from the group  
20 consisting of:

substantially the same amino acid sequence set forth in SEQ ID NO:46; and

substantially the same sequence of amino acid residues 1 through 56 of an HIV-1 Nef  
25 protein wherein amino acid residue 54 is aspartic acid.

9. The truncated protein of claim 8 wherein said amino acid sequence is selected from the group consisting of:

5 amino acid residues 1 through 56 of SEQ ID NO:46; and

amino acid residues 1 through 56 of an HIV-1 Nef peptide wherein amino acid residue 54 is aspartic acid.

10. An antibody or fragment thereof that  
10 specifically binds to the mutated protein of claim 7, 8 or 9.

11. A substantially pure nucleic acid molecule or fragment thereof that encodes a mutated HIV-1 Env gp41 protein containing an arginine at amino acid residue 660.

15 12. The nucleic acid molecule of claim 11 wherein nucleotide 1979 is guanine.

13. A substantially pure fragment of the nucleic acid molecule of claim 12 comprising a portion of said nucleic acid molecule that contains said guanine at  
20 nucleotide 1979 and can be used to specifically detect the nucleic acid molecule of claim 12.

14. A substantially pure mutated HIV-1 Env gp41 protein fragment, comprising a peptide encoded by the nucleic acid molecule fragment of claim 13.

25 15. An antibody or fragment thereof that specifically binds to the protein fragment of claim 14.

16. A substantially pure mutated HIV-1 *Env* gp41 protein or fragment thereof comprising an HIV-1 *Env* gp41 protein containing an arginine at amino acid residue 660.

5 17. An antibody or fragment thereof that specifically binds to the protein of claim 16.

18. A substantially pure nucleic acid molecule or fragment thereof that encodes a mutated HIV-1 *Env* gp120 protein selected from the group consisting of:

10 (a) a nucleic acid sequence that encodes an HIV-1 *Env* gp120 protein wherein:

amino acid residue 143 is either serine or arginine;

15 up to eight amino acids at amino acid residues 144 through 151 in the V1 domain are deleted; and

amino acid residue 153 is either methionine or isoleucine; and

20 (b) a nucleic acid sequence that encodes an HIV-1 *Env* gp120 protein wherein:

amino acid residue 187 is either isoleucine or valine; and

25 one or two amino acids at amino acid residues 192 and 193 in the V2 domain are deleted.

19. A substantially pure fragment of the nucleic acid molecule of claim 18 comprising a portion of the nucleic acid molecule that contains said deletions and that can be used to specifically detect the nucleic acid molecule of claim 18.

20. A substantially pure mutated HIV-1 *Env* gp120 protein fragment, comprising a peptide encoded by the nucleic acid molecule fragment of claim 19.

21. An antibody or fragment thereof that specifically binds to the protein fragment of claim 20.

22. A substantially pure mutated HIV-1 *Env* gp120 protein or fragment thereof selected from the group consisting of:

(a) the amino acid sequence of an HIV-1 *Env* gp120 protein wherein amino acid residue 143 is either serine or arginine; up to eight amino acids at amino acid residues 144 through 151 in the V1 domain are deleted; and amino acid residue 153 is either methionine or isoleucine; and

(b) the amino acid sequence of an HIV-1 *Env* gp120 protein wherein amino acid residue 187 is either isoleucine or valine; and one or two amino acids at amino acid residues 192 and 193 in the V2 domain are deleted.

23. An antibody or fragment thereof that specifically binds to the protein of claim 22.

24. A substantially pure nucleic acid molecule or fragment thereof that encodes a truncated HIV-1 *Vpr* protein comprising a nucleic acid sequence encoding substantially the same amino acid residues 1 through 17 of an HIV-1 *Vpr* protein.

25. The nucleic acid molecule of claim 24 wherein said nucleic acid sequence comprises nucleotides 1 through 51 of an HIV-1 *vpr* gene.

26. A substantially pure truncated HIV-1 *Vpr* protein or fragment thereof comprising substantially the same amino acid residues 1 through 17 of an HIV-1 *Vpr* protein.

27. An antibody or fragment thereof that specifically binds to the protein of claim 26.

28. A substantially pure nucleic acid molecule or fragment thereof that encodes a mutated and truncated HIV-1 *Pol* protease protein comprising a nucleic acid sequence encoding substantially the same sequence of amino acid residues 1 through 13 of a wild-type HIV-1 *Pol* protease protein and amino acid residues 14 through 29 of SEQ ID NO:6.

29. The nucleic acid molecule of claim 26 wherein said nucleic acid sequence comprises nucleotides 1 through 41 of a wild-type HIV-1 *Pol* protease gene and nucleotides 42 through 87 of SEQ ID NO:5.

30. A substantially pure fragment of the nucleic acid molecule of claim 29, comprising a fragment of SEQ ID NO:5 at least 8 nucleotides in length and that can be used to specifically detect the nucleic acid molecule of claim 29.

31. A substantially pure mutated and truncated HIV-1 *Pol* protease protein or fragment thereof comprising substantially the same sequence of amino acid residues 1 through 13 of a wild-type HIV-1 *Pol* protease protein and amino acid residues 14 through 29 of SEQ ID NO:6.

32. An immunogen comprising an inactivated protease-defective viral HIV-1 particle containing one or more of the following substantially pure proteins:

an HIV-1 *Env* gp120 protein comprising substantially the same protein of claim 22;

a mutated HIV-1 *Pol* protease molecule comprising substantially the same sequence of amino acid residues 1 through 13 of a wild-type HIV-1 *Pol* protease protein and amino acid residues 14 through 29 of SEQ ID NO:6;

a truncated HIV-1 *Nef* protein or fragment thereof comprising substantially the same sequence of amino acid residues 1 through 56 of an HIV-1 *Nef* protein;

a mutated HIV-1 *Nef* protein comprising substantially the same amino acid sequence set forth in SEQ ID NO:46 or a fragment thereof comprising one or more of the following amino acid residues of SEQ ID NO:46: alanine at amino

55

acid residue 15, lysine at amino acid residue 19, valine at amino acid residue 33, and aspartic acid at amino acid residue 54;

5 a truncated HIV-1 *Nef* protein comprising substantially the same sequence of amino acid residues 1 through 56 of a *Nef* peptide wherein amino acid residue 54 is aspartic acid;

10 a truncated HIV-1 *Vpr* protein or fragment thereof comprising substantially the same amino acid residues 1 through 17 of an HIV-1 *Vpr* protein; or

15 an HIV-1 *Env* gp41 protein or fragment thereof comprising substantially the same amino acid sequence of an HIV-1 *Env* gp41 protein containing an arginine at amino acid residue 660.

33. The immunogen of claim 32 further comprising one or more suitable adjuvants.

20 34. A method of detecting a mutated HIV-1 gene selected from the group consisting of a *nef* gene, an *env* gp41 gene and an *env* gp120 gene or fragments thereof in a sample of cells, lysed cells or extracellular fluid from an individual, comprising the steps of:

25 obtaining from an individual a sample of cells, lysed cells or extracellular fluid suspected of containing said mutated gene or fragments thereof;

contacting said sample with a measured amount of the nucleic acid molecule of claims 4, 13 or 19, respectively, to hybridize with said gene or fragment thereof; and

5                   determining the presence of said hybridized molecule to detect the presence of the mutated gene or fragments thereof in said sample.

35. A method of detecting the presence of a  
10 mutated HIV-1 protein selected from the group consisting of a *Nef* protein, an *Env* gp41 protein and an *Env* gp120 protein or fragments thereof in an individual, comprising the steps of:

15                   obtaining from an individual a sample of cells, lysed cells or extracellular fluid suspected of containing a mutated HIV-1 protein or fragments thereof;

20                   contacting said sample with a measured amount of the antibody of claims 6 or 10, 15 or 17, or 21 or 23, respectively, to form a complex of the antibody and said protein or fragment thereof; and

25                   determining the presence of said complex to detect the presence of the mutated protein or fragments thereof in said sample.

36. A method of reducing the severity of HIV-1 infections in individuals, comprising contacting protease-defective viral particles containing one or more mutated HIV-1 Nef proteins, mutated HIV-1 Env gp41  
5 proteins, or mutated HIV-1 Env gp120 proteins or fragments thereof present on cells, in lysed cells or in extracellular fluid with an effective amount of the antibody of claims 6 or 10, 15 or 17, or 21 or 23, respectively, to inhibit the activity of said proteins or  
10 fragments thereof.

37. The method of claim 36 wherein said antibody is attached to a moiety selected from the group consisting of radioactive moieties, chemotherapeutic moieties and chemotoxic moieties.

15 38. A method of reducing or preventing apoptotic cell lysis in an HIV-1 seropositive or seronegative individual, comprising administering an immunologically effective amount of the immunogen of claim 32 or 33 to the individual.

FIGURE 1

## nucleotide

40  
pol prot. LAI AA-GATAGGGGGCAACTAAAGGAGCTCTATTAGATACAGGAGCAGATGAT SEQ. ID NO. 1  
K I G G Q L K E A L L D T G A D D SEQ. ID NO. 2  
MO/LAI ..... SEQ. ID NO. 3  
..... SEQ. ID NO. 4  
L-2 ..T..... SEQ. ID NO. 5  
N D R G A T K G S S I R Y R S R \* SEQ. ID NO. 6  
14 30  
amino-acid residue

## nucleotide

1  
vpr LAI ATGGAACAAGCCCCAGAGACCAAGGGCCACAGAGGGAGCCACACAATGAATGGACACTA SEQ. ID NO. 7  
M E Q A P E D Q G P Q R E P H N E W T L SEQ. ID NO. 8  
MO/LAI .....A..... SEQ. ID NO. 9  
.....A..... SEQ. ID NO. 10  
L-2 .....A..... SEQ. ID NO. 11  
.....A..... SEQ. ID NO. 12  
1 18  
amino-acid residue

## nucleotide

424  
env gp120 LAI AGTAGTAATACCAATAGTAGTAGCGGGGGAATGATG SEQ. ID NO. 13  
S S N T N S S S G E M M SEQ. ID NO. 14  
MO/LAI .....A..... SEQ. ID NO. 15  
- R # # # # # # # # # # I SEQ. ID NO. 16  
.....A..... SEQ. ID NO. 17  
- R # # # # # # # # # # I SEQ. ID NO. 18  
142 153  
amino-acid residue

558 582  
ATAATACCAATAGATAATGATACTACC SEQ. ID NO. 19  
I I P I D N D T T SEQ. ID NO. 20  
.....G..... SEQ. ID NO. 21  
- V - - # # - SEQ. ID NO. 22  
.....G..... SEQ. ID NO. 23  
- V - - # # - SEQ. ID NO. 24  
186 194

**FIGURE 1 CONT.**

nucleotide		1989		1951		1989	
env gp41	LAI	ATTGAAGATCGCAATACCAAGCAAGAAAGATGACAA	SEQ. ID NO. 25	ATTGAAGATCGCAATACCAAGCAAGAAAGATGACAA	SEQ. ID NO. 26	ATTGAAGATCGCAATACCAAGCAAGAAAGATGACAA	SEQ. ID NO. 27
	MO/LAI	I E E S Q N Q Q E K N E Q	SEQ. ID NO. 28	I E E S Q N Q Q E K N E Q	SEQ. ID NO. 29	I E E S Q N Q Q E K N E Q	SEQ. ID NO. 30
	L-2	.....G.....	SEQ. ID NO. 31	.....G.....	SEQ. ID NO. 32	.....G.....	SEQ. ID NO. 33
		- - - - - R - - -	SEQ. ID NO. 34	- - - - - R - - -	SEQ. ID NO. 35	- - - - - R - - -	SEQ. ID NO. 36
		651		651		651	
	amino-acid residue						
nucleotide							
		130		130		130	
nef	LAI	ACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGGCTGCTAGAGCA	SEQ. ID NO. 37	ACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGGCTGCTAGAGCA	SEQ. ID NO. 38	ACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGGCTGCTAGAGCA	SEQ. ID NO. 39
	MO/LAI	T S S N T A A T N A A C A W L E A	SEQ. ID NO. 40	T S S N T A A T N A A C A W L E A	SEQ. ID NO. 41	T S S N T A A T N A A C A W L E A	SEQ. ID NO. 42
	L-2	.....A.....	SEQ. ID NO. 43	.....A.....	SEQ. ID NO. 44	.....A.....	SEQ. ID NO. 45
		- - - - - A - - -	SEQ. ID NO. 46	- - - - - A - - -	SEQ. ID NO. 47	- - - - - A - - -	SEQ. ID NO. 48
		44		44		44	
	amino acid residue						
nucleotide							
		180		180		180	
nef	LAI	ACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGGCTGCTAGAGCA	SEQ. ID NO. 49	ACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGGCTGCTAGAGCA	SEQ. ID NO. 50	ACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGGCTGCTAGAGCA	SEQ. ID NO. 51
	MO/LAI	T S S N T A A T N A A C A W L E A	SEQ. ID NO. 52	T S S N T A A T N A A C A W L E A	SEQ. ID NO. 53	T S S N T A A T N A A C A W L E A	SEQ. ID NO. 54
	L-2	.....A.....	SEQ. ID NO. 55	.....A.....	SEQ. ID NO. 56	.....A.....	SEQ. ID NO. 57
		- - - - - A - - -	SEQ. ID NO. 58	- - - - - A - - -	SEQ. ID NO. 59	- - - - - A - - -	SEQ. ID NO. 60
		57		57		57	
	amino acid residue						

FIGURE 2

PRIMERS USED FOR PCR AMPLIFICATION OF HIV-1 PROVIRUS IN L-2						
Primer		Nucleotide No.	Sequence	Target gene(s)	Amplified size (bp)	SEQ ID NO.
gag-d	Sense	(1921-1942)	5' CAGAAAGGCAATTTTAGGAACC 3'			37
	Antisense	(2590-2571)	5' CCTGGCTTTAATTTTACTGG 3'	pol (protease)	670	38
pol-3	Sense	(4808-4826)	5' GTACAGGGGAAAGAATA 3'			39
env-3	Antisense	(6341-6317)	5' CCCCATATAAGACTGTGACCCACAA 3'	vif, vpr, vpu	1538	40
EN-D-Sal	Sense	(6221-6238)	5' TGAGTCGACATGAGAGTGAAGGAGAAATAT 3'			41
EA-Pst	Antisense	(8782-8763)	5' TCCTGCAGCTTATAGCAAAATCCTTTCCA 3'	env (gp120, gp41)	2562	42
EN53	Sense	(8655-8678)	5' TCAATGCCACAGCCATAGCAGTAG 3'			43
LR3	Antisense	(9588-9568)	5' CAGTGGGTTCCCTAGTTAGCC 3'	nef	934	44

**FIGURE 3**

SEQ. ID NO. 45

1	11	21	31	41
ATGGGTGGCA	AGTGGTCAAA	AAGTAGTGTG	GTTGGATGGC	CTGCTGTAAG
51	61	71	81	91
GGAAATG	ACCGAGCTG	AGACAGCAGC	AGATGGGGTG	GGAGCAGTAT
101	111	121	131	141
CTCGAGACCT	A GAAAAA CAT	GGAGCAATCA	CAAGTAGCAA	TACAGCAGCT
151	161	171		
ACCAATGCTG	ATTGTGCCTA	GCTAGAAGCA		
181	191	201		
CAAGAGGAGG	AGGAGGTGGG	TT - - -		

**FIGURE 4****SEQ. ID NO. 46**

(amino) - NH<sub>2</sub> - MGGKWSKSSV VGWF **A** VRE **K** MRRRAEPAADGV GA **V** SRDLEKH GAIT  
SSNTAA TNA **D** CA - COOH (carboxy)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/06690

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/139.1, 148.1, 160.1, 188.1, 205.1, 208.1; 435/5, 6, 236, 237; 436/504; 530/387.9, 388.35, 389.4, 395, 403; 536/23.72.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	KAMEOKA et al. Induction of apoptosis by protease-defective particle preparations of human immunodeficiency virus type 1 is specific to a subset of U937-derived subclones. International Immunology. 1996. Vol. 8, No. 11, pages 1687-1697, see entire document.	32 ----- 1-31, 33-38
X, P ----- Y, P	BAHMANI et al. Production of Doughnut-shaped, protease-defective particles from a human T cell clone carrying a provirus with specific mutations in the env, pol, vpr, and nef genes. AIDS Research and Human Retroviruses. 1997. Vol. 13, No. 6, pages 523-526, see entire document.	1-5, 7-9, 11-14, 16, 18-20, 22, 24- 26, 28-32 ----- 6, 10, 15, 17, 21, 23, 27, 33-38



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

22 JUNE 1998

Date of mailing of the international search report

18 AUG 1998

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/06690

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P ----- Y, P	KAMEOKA et al. Exposure of resting peripheral blood T cells to HIV-1 particles generates CD25+ killer cells in a small subset, leading to induction of apoptosis in bystander cells. International Immunology. 1997. Vol. 9, No. 10, pages 1453-1462, see entire document.	32 ----- 1-31, 33-38
X -- Y	OTAKE et al. The carboxyl-terminal region of HIV-1 nef protein is a cell surface domain that can interact with CD4+ T cells. The Journal of Immunology. 1994. Vol. 153, pages 5826-5837, see Abstract.	6, 10 ----- 35
Y	KISHI et al. Naturally occurring accessory gene mutations lead to persistent human immunodeficiency virus type 1 infection of CD4-positive T cells. Journal of Virology. December 1995. Vol. 69, No. 12, pages 7507-7518, see entire document.	24-27
Y	WANG et al. Particle assembly and Vpr expression in human immunodeficiency virus type 1-infected cells demonstrated by immunoelectron microscopy. Journal of General Virology. 1994. Vol. 75, pages 2607-2614, see entire document.	24-27
X --- Y	KAMEOKA et al. Protease-defective, gp120-containing human immunodeficiency virus type 1 particles induce apoptosis more efficiently than does wild-type virus or recombinant gp120 protein in healthy donor-derived peripheral blood T cells. Journal of Clinical Microbiology. January 1997. Vol. 35, No. 1, pages 41-47, see entire document.	32 ----- 1-31, 33-38

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/06690

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐  
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 39/21, 39/42; C07H 21/02, 21/04; C07K 14/155, 16/10; C12N 7/04, 7/08; C12Q 1/68, 1/70; G01N 33/577, 33/60, 33/68.

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/139.1, 148.1, 160.1, 188.1, 205.1, 208.1; 435/5, 6, 236, 237; 436/504; 530/387.9, 388.35, 389.4, 395, 403; 536/23.72.

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-5, 7-9 and 34, truncated nef proteins and nucleic acids and method of use.

Group II, claim(s) 6, 10 and 35, drawn to antibodies to mutated nef protein.

Group III, claim(s) 11-14, 16 and 34, drawn to mutated gp41 proteins and nucleic acids and method of use.

Group IV, claim(s) 15, 17 and 35, drawn to antibodies to mutated gp41 and method of use.

Group V, claim(s) 18-20, 22 and 34, drawn to mutated gp120 proteins and nucleic acids and method of use.

Group VI, claim(s) 21, 23 and 35, drawn to antibodies to mutated gp120 and method of use.

Group VII, claim(s) 24-26, drawn to mutated vpr proteins and nucleic acids.

Group VIII, claim(s) 27, drawn to antibodies to mutated vpr.

Group IX, claim(s) 28-31, drawn to mutated pol proteases and nucleic acids.

Group X, claim(s) 32-33 and 38, drawn to defective HIV-1 particles and method of use.

Group XI, claim(s) 36-37, drawn to method of treating HIV-1 using antibodies.

The inventions listed as Groups I-XI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the products of Groups I-X are directed to different mutated proteins, antibodies and nucleic acids which differ in their physical and chemical structures such as primary sequence and immunologic activity and are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general inventive concept. Further, the methods of Groups I-VI and X-XI differ in their industrial applicability, reagents and method steps and are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general inventive concept.